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Biocontrol Potentiality of Active Ingredients from Endophytic *Bacillus subtilis* Isolated from *Alhagi pseudalhagi* Desv on Maize Spot Diseases

Mahmutjan Dawut[1,3], Abdukadir Abliz[1,2], Wenera Ghopur[1], Pezilet Behti[1], Burabiyem Obulkhasim[1] and Ghopur Mijit[1]*

[1]*College of Life Science and Technology, Xinjiang University, China*
[2]*Media Institute of Urumqi Vocational University, China*
[3]*Graduate School of Science, Hokkaido University, Japan*

Abstract

Two endophytic bacterial strains, XJAS-AB-13 and XJAS-AB-11, with broad antifungal activity against maize spot pathogens *Exserohilum turcicum* and *Bipolaris maydis*, were screened from the *Alhagi pseudalhagi* Desv, by using agar diffusion method. Both of them were identified as *Bacillus subtilis* according to the physiological and biochemical properties, as well as the molecular analysis based on 16S rDNA sequence. The inhibitory activity of broth cultured XJAS-AB-13 and XJAS-AB-11, measured *in vivo*, and the result showed that the diseases inhibition efficiency of XJAS-AB-13 and XJAS-AB-11 broth on *E. turcicum* and *B. maydis* reached 63.33%, 45.0% and 23.33%, 58.34% respectively. Infection by different diseases caused an increase in total amount of the protein in general, where the increment generated by the *Exserohilum turcicum* was greater than *Bipolaris maydis.* Defense enzymes activity of Superoxide dismutase (SOD), however, declined sharply compare to the control group, while the others, catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX), were increasing. Through extraction and silica-gel chromatography, two different monomers, XJAS-B and XJAS-G, were isolated from the ethyl acetate extraction of the culture broth of XJAS-AB-11, and identified according to ^{1}HNMR and ^{13}CNMR spectral data as well as ESI-MS molecular weight analysis as cyclo-(D-leucyl-trans-4-hydroxy-L-proline) [(3R,7R,8aS)-7-hydroxy-3-isobutylhexahydropyrrolo [1,2-a] pyrazine-1,4-dione] and 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one. The preliminary comparative analysis on XJAS-AB-11 Naringenin-chalcone synthase (*CHS*) gene also indicated their high homology with some prokaryotic and eukaryotic *CHS* amino acid sequences. In addition, the amino acid sequence of XJAS-AB-11 *CHS* gene has showed similar amino acid composition with those of *Streptomyces griseus*, *Arachis hypogaea* and *Gerbera hybrida* at the active site.

Keywords: Antagonistic endobacteria; *In vivo* disease control test; Active monomers; Structure identification; Chalcone synthase

Introduction

Maize is one of the most essential cereal crops used in the human diet in most parts of the world and it is vital constituent for animal feeds. Among cereal crops, maize remains third after wheat and rice in total area and production in the world. Despite the huge production and consumption ratio of the maize, diseases caused by different pathogens were becoming worse recently. The damage of main cereal crops around the world is up to 10% to 15%, even if number of different precautions had taken, where 70% to 80% of which caused by pathogenic fungi [1,2]. Traditionally, diseases are prevented using chemical disinfectants, but this practice is considered unsustainable and harmful due to their potential threat towards natural environment and human health [3,4]. Thus, developing a new bio-pesticide, which is non-toxic and non-polluting, is attaining increasing interests among researchers [5-8].

An 'endophyte', a concept which was put forward by Barry in 1866, is a microorganism living in the tissue of a healthy plant, but does not cause any harm to the host [9]. Endophytes include fungi, actinomycetes, and bacteria; these can be found in any different parts of host plants, such as in roots, flowers, fruits, and seeds [10,11]. The secondary metabolites produced by endophytes, especially by those living in the medicinal plants could be the abundant source of new chemical compounds which is essential not only to human health but also for the prevention and treatment of different diseases among the animals and plants [12-15].

The traditional medicinal plant *Alhagi pseudalhagi* Desv is mainly distributed in the flat saline land of Badain Jaran Desert in inner Mongolia, Xinjiang, and Gansu province in China. *Alhagi pseudalhagi* Desv have not been only used as a sand fixing plant in Xinjiang but is also been used as an effective natural traditional medicine particular in curing joint pain, detoxification [16]. Although, there are several studies on screening of active secondary metabolites of *A. pseudalghi*, and resulted to the isolation and identification of 15 different compounds [17]. However, there is no report on screening and testing of active secondary metabolites of endophytic microorganisms isolated from Xinjiang *A. pseudalghi in vivo* level. For the first time, we report herein the result of our study on isolating secondary metabolites from an *A. pseudalghi*-endophytic microorganism and the compound's activity against pathogenic organisms associated with maize spot disease.

Materials and Methods

Strains and cultures

The medicinal plant *Alhagi pseudalhagi* Desv was collected from Xinjiang Turpan, in 2010.

The plant pathogens, *Fusarium oxysporum f.sp. cucumerinum*, *Fusarium oxysporum f.sp. vasinfectum*, *Fusarium oxysporumf.sp. niveum*, *Alternaria mali*, *Exserochilum turcicum*, *Bipolaris maydis*,

***Corresponding author:** Ghopur Mijit, Plant Cell Biology Laboratory of The College of Life Science and Technology, Xinjiang University, Xinjiang 830046, China, Tel: 086-0991-8583452; E-mail: gmijit@163.com

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Bacillus subtilis were purchased from the culture collection center of the institute of agricultural resources and regional planning of Chinese academy of agricultural sciences (CAAS); the animal pathogens, *Staphylococcus aureus* and *Escherichia coli*, were kindly provided by microbiology lab of the college of life science and technology, Xinjiang University.

Escherichia coli DH5α was purchased from TransGene Biotech Company. The traditional maize seed without any genetic modification (No. SC-704) was purchased from Xinjiang academy of agricultural science.

The culture medium LB (Luria Broth), MEA (Malt Extract Agar), and PDA (Potato Dextrose Agar) used in this study were prepared according to Qian et al. [18].

Screening and identification

After washing those fresh leaves and stems, which are obtained from the newly collected medicinal plant *Alhagi pseudalhagi* Desv with tap water, the surface was sterilized according to Geris dos Santos [19]; and then grinned it in sterilized mortar and filtered with gauze [20]. The filtrate was evenly coated on the different culture medium to grow. Endophytic microorganisms grown in their culture mediums were purified according to their colony color and morphological treats, gradually.

The fermentation broth of isolated endophytic fungi and bacteria were prepared under the condition of 28℃ (fungi), 37℃ (bacteria), 150 r/min agitation, for 96 h and 72 h, respectively. The inhibitory activity of isolated microorganism`s broth on the different plant and animal pathogens were measured via agar diffusion method [21], and the inhibition zone diameter was gauged. Then, the morphological characteristics of endophytes, which are showed remarkable effects on pathogens described according to Buchanan and Dong et al. [22,23]. Plus, transmission electron microscope (TEM; H-600, Japan) was also used for more detailed morphological analyses.

For the molecular analyses, the total genomic DNA of selected endophytes was extracted by UNIQ-10 column bacterial genomic DNA extraction Kit (Sangon Biotech) and 16S rDNA region was sequenced using the bacterial universal primer 27F and 1492R.

Disease prevention *in vivo* test

The test plant seeds, SC-704, was planted in the nutrition bottle which contains of 3:1 sill/perlite, and irrigated with 1/2, 1/4 fold of MS nutrition solution; the cultivation room temperature was controlled at 25℃.

Since two leaf stage, maize seedlings were separated into seven groups (each group contains 30 maize seedling, with a total of 60 leaves per group): one blank control group (no any treatment); two negative control groups (only treated with *E. turcicum* and *B. maydis* spore suspension, 50 spore/40 uL); two treatment groups (after treated with fermentation broth, next day treated with *E. turcicum* and *B. maydis* spore suspension, respectively); two positive control groups (first day, treated with 0.1% Carbendazol (broadly used pesticide), next day treated with *E. turcicum* and *B. maydis*). Observation of the result and calculation of disease prevention efficiency were done 10 days after.

Just after finishing up the observation of disease prevention efficiency, the same seven group subjected to measurement of total protein content, and variation on the activity of 4 different disease control related defense enzymes, superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX), according to Chen et al. [24]. One-way ANOVA analysis, at α=0.05 and α=0.01, was applied for further statistical analysis.

Isolation and structural analysis of XJAS-AB-11 metabolites

Fifth day of culturing the strain XJAS-AB-11 in LB medium at 37℃, collected the broth supernatant by centrifuging, then used it for extraction by four different chemical solutions, isopyknic petroleum ether, normal butyl alcohol, chloroform, ethyl acetate. This extraction proceeded until the extracts became colorless. Then, the bacteriostatic effect of those extracts against different plant/animal pathogens were examined by adopting the same method mentions earlier and the aqueous methanol (5%) as a negative control. After determined the active site of the culture broth of XJAS-AB-11, the bulk fermentation and its extraction took placed.

Thin layer chromatographic (TLC) pre-test was applied for the purpose of choosing the optimal developing solvent for the use in the silica gel column chromatography. Took a rectangular silica plate (type GF254, 5 × 10 cm) draw two horizontal lines at the 0.5 cm from the bottom and the top, used one of which (bottom) as the spotting line or the baseline, others as end line. After dissolving the extract in methanol, inhaled it into 0.33 mm capillary tube and gently put a dot on the spotting line. Total of three developing solvent with distinct polarity were used, which are petroleum ether/acetone, Petroleum ether/ethyl acetate, chloroform/methanol, as developer. After pulled the plate out from the urn, dried it naturally at the room temperature and visualized it by sulfuric acid-ethanol solution, then observed it under UV-analyzer, at 254 nm and 365 nm wavelength, and the retention value (R_f=distance from baseline travelled by solute/distance from baseline travelled by solvent) was calculated (considered the value between 0.2-0.8 is the best).

The silica gel column chromatography of elected extract was done through gradient eluting by different proportion of chloroform: methanol (100:1, 70:1, 50:1, 30:1, 10:1, 7:1, 5:1, 1:1). The eluents were collected into test tubes in the order of from weak polarity to strong. TCL test was applied on each test tube, and concentrated them in vacuum. The ^{1}HNMR and ^{13}CNMR, API-ES mass spectrometer and infrared spectrogram were applied to identify the structure of the isolated pure monomers.

Preliminary study on the Naringenin-Chalcone synthase (*CHS*) gene

Obtaining *CHS* partial sequence: after extracting the genomic DNA using the purchased UNIQ-10 bacterial DNA extraction kit, the *CHS* partial gene sequence was amplified by using two pairs of primers: *B. subtilis* bcsA-ypbQ operon *CHS* gene primer (BpsA-1 and BpsA-2) [25] and the newly designed primer by our laboratory according to *B. subtilis* naringenin-chalcone synthase gene (Bsn5_01495) sequence (accession No. YP_004203960.1) Bsn-1: 5′-AGCGGCTGTTCGTGAGTGCC-3′ and Bsn-2: 5′-CCCGGCCCTAATGCGCCAAT-3′. The PCR amplification was conducted in 50 μm reaction system, which includes distilled water (31 μm), PCR buffer (with Mg^{2+}, 5 μm), Deoxynucleotide (dNTP, 4 μm), each primer (2 μm), template DNA (5 μm) and Taq enzyme (1.0 μm). The reaction was done with 35 cycle (denatured at 94℃ for 60s, annealing at (Bsn/58.4, BpsA/66.0) for 90s, extension at 72℃ for 2 min), which was initialized at 94 for 5 min, and elongated 72 for 10 min, finally terminated at 4℃. After obtaining the target sequences, the sequence amplified by *B. subtilis* bcsA-ypbQ operon CHS gene primer was ligated to the Invitrogen TA cloning vector, and then transformed it into *E. coli* competent cell, and sequenced the vector. DNAMAN was applied for the purpose of multiple comparisons

between the DNA sequences of Bsn and BpsA (the *B. subtilis* BSn5 naringenin-chalcone synthase was selected as standard sequence).

Contrastive analysis of XJAS-AB-11 CHS partial sequence: GenBank blast tools (http://www.ncbi.nlm.nih.gov/) were used to compare similar CHS gene and amino acid sequences. The construction of the secondary and three-dimensional structure of enzyme was done by SWISS-model molecular modeling system (http://swissmodel.expasy.org/), and NRPS-PKS database (http://nrps.igs.umaryland.edu/) was applied to searching of active site amino acid sequence of CHS enzyme from different species. The CHS amino acid sequences comparison analysis was done through online ClustalW 2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Results

Morphology and identity of two antagonistic bacteria

Total of 14 endophytic bacteria and 6 endophytic fungi strains were isolated from *A. pseudalhagi* Desv. Among which the bacteria XJAS-AB-13 and XJAS-AB-11 showed much stronger and stable inhibitory effect especially on the *B. maydis* (Tables 1 and 2). The inhibition zone average diameters of XJAS-AB-13 on the *E. turcicum* and *B. maydis* up to 24.6 mm and 32.8 mm, respectively. As mentioned in our previous work, XJAS-AB-11 had showed strong inhibitory effect on both *E. turcicum* and *Alternaria mali*, the effect on the former was much stronger (31.5 mm) than the latter [26]. Both bacterial strains, XJAS-AB-13 and XJAS-AB-11, forms circular colony, and cells were rhabditiform, colonies were slimy and jagged, has ivory gloss on surface, and easy to pick up (Table S1 and Figure S1).

The phylogenetic tree inferred from 16S rDNA sequence (Figure S2) demonstrated the high homology between the XJAS-AB-13 and *Bacillus subtilis* BS-HOT1 (99%). So, based on the molecular and morphological characteristics, XJAS-AB-13 identified as *B. subtilis* and registered into GeneBank (registration number is JF826131) while strain XJAS-AB-11was identified as a subspecies of *B. subtilis* in our previous work [26].

In vivo test and determination of defense enzyme activity

For XJAS-AB-13, all leaves were growth normally without any infection in the blank control group. In the negative control group, however, almost all leaves were infected except 9 of them, the infection rate of *E. turcicum* and *B. maydis* up to 85% and 76.67%. Yet there was no significant difference between the infection rate of *E. turcicum* and *B. maydis*. But the significant difference ($P<0.01$) was occurred only between the infection rate of the treatment group and its relative negative control group (Table 1 and Figure S3). This also explains that the culture broth of XJAS-AB-13 is more efficient on preventing *E. turcicum* than *B. maydis*.

As shown in the Table 2 and Figures S4-S6, all leaves were growth healthy in blank control group, whereas the 85% (51 leaves) and 76.67% (46 leaves) of the leaves were infected in the negative control group. The statistical significant difference ($P<0.01$) between the treatment group and its relative negative control group of *B. maydis* showed the strong inhibitory efficiency of the XJAS-AB-11 culture broth on *B. maydis*, while its control rate only up to 23.33% on *E. turcicum*.

In general, the infection by diseases increased the production of different proteins in both cases (AB-11and AB-13). As shown in the Figure 1, infection without any preventing protocols caused increase in different proteins (Figure 1, NC 36260 and NC36265). However, application of AB-13 culture broth decreased the total protein content of the leaves sharply (Figure 1, T 36260 and T 36265). The multiple comparison result showed significant differences ($\alpha=0.01$) between each pair of group, except between the blank control and negative control group.

On the contrary, there was no significant difference between negative control group and treatment group of *E. turcicum*, although there was a significant difference ($\alpha=0.01$) between negative control group and treatment group of *B. maydis* (Figure 2).

The changes among the enzyme activity of four different enzymes were varies greatly. As shown in the Figure 3A, disease infection by both *E. turcicum* and *B. maydis* distinctly ceased the enzyme activity of SOD, especially of the negative control group. However, the exposure to the culture broth of XJAS-AB-13 kept the activity of SOD almost the same with blank control group, and there is no significant difference between them ($P<0.01$) (Figure 3A). Enzyme activity of POD and CAT

Pathogen inoculated	Treating type	Inoculated	Infected	Infection rate	Normal	Control rate
Exserohilum turcicum	Treated	60	13	21.67%	47	63.33%**
Exserohilum turcicum	Negative control	60	51	85%	9	—
Exserohilum turcicum	Positive control	60	4	6.67%	56	78.33%
No pathogen inoculated	Blank	0	0	0	60	—
Bipolaris maydis	Negative control	60	46	76.67%	14	—
Bipolaris maydis	Positive control	60	4	6.67%	56	70%
Bipolaris maydis	Treated	60	19	31.67%	41	45%**

Note: In the table, "**" means that between that treatment group and its relevant negative control group has significant difference ($P<0.01$)

Table 1: Controlling efficiency of XJAS-AB-13 culture broth on *Exserochilum turcicum* and *Bipolaris maydis*.

Pathogen inoculated	Treating type	Inoculated	Infected	Infection rate	Normal	Control rate
Exserohilum turcicum	Treated	60	37	61.67%	23	23.33%
Exserohilum turcicum	Negative control	60	51	85%	9	—
Exserohilum turcicum	Positive control	60	4	6.67%	56	78.33%
No pathogen inoculated	Blank	0	0	0	60	—
Bipolaris maydis	Negative control	60	46	76.67%	14	—
Bipolaris maydis	Positive control	60	4	6.67%	56	70%
Bipolaris maydis	Treated	60	11	18.33%	49	58.34%**

Note: in the table, "**" means that between that treatment group and relevant negative control group has significant difference ($P<0.01$)

Table 2: Controlling efficiency of XJAS-AB-11 culture broth on *Exserochilum turcicum* and *Bipolaris maydi.*

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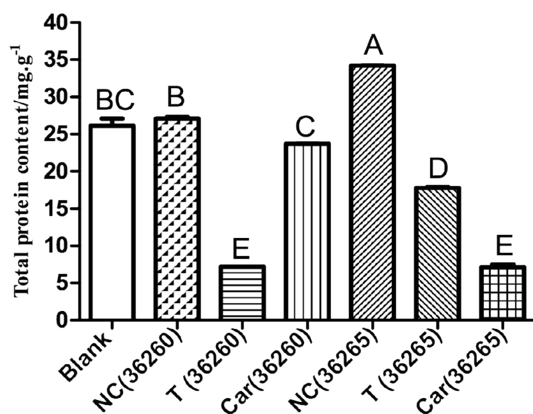
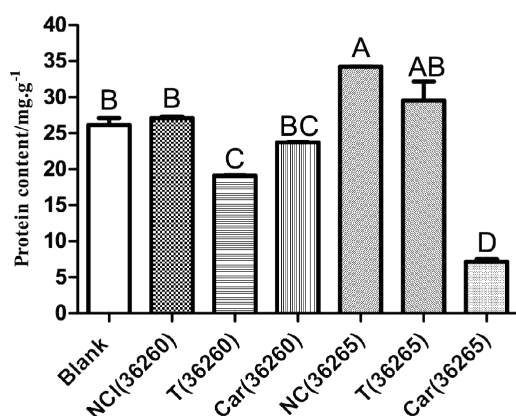


Note: Blank (Blank group); NC (Negative Control group); T (Treatment group with XJAS-AB-13); Car (Positive Control group or Carbendazim treated group). (36265) and (36260) represents *Exserochilum turcicum* and *Bipolaris maydis*, respectively. Different capital letters represents there has significant difference in total protein content of two groups (α=0.01).

Figure 1: Total protein content of each group (XJAS-AB-13).



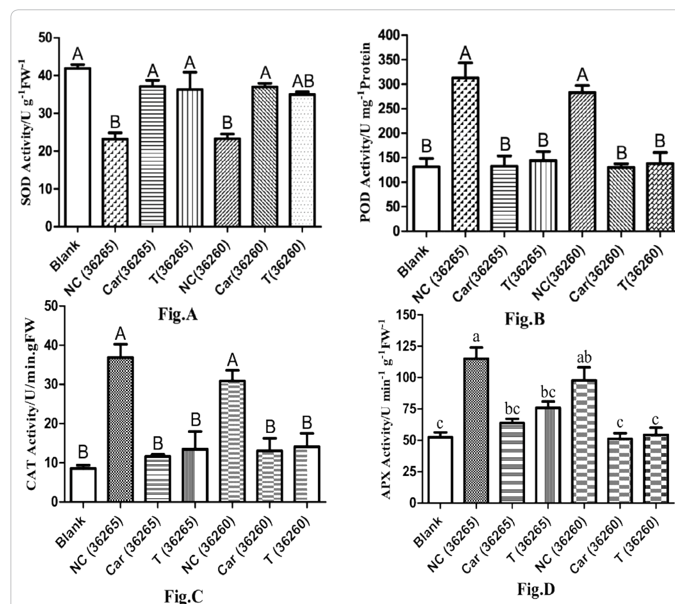
Note: Blank (Blank group); NC (Negative control group); T (Treatment group with XJAS-AB-11); Car (Positive control group or Carbendazim treated group). (36265) and (36260) represents *Exserochilum turcicum* and *Bipolaris maydis*, respectively. Different capital letters represents there has significant difference in total protein content of two groups (α=0.01).

Figure 2: Total protein content of each group (XJAS-AB-11).

among the treatment group (with AB-13) and positive control group was significantly lower than negative control group, approximately 2.162 and 2.737 time lower that negative control group (Figure 3). For XJAS-AB-11 (Figure 4), a similar SOD activity change with XJAS-AB-13 was observed. However, after exposure to pathogens and AB-11 fermentation broth, in general, all of CAT, POD and APX showed increasing tendency.

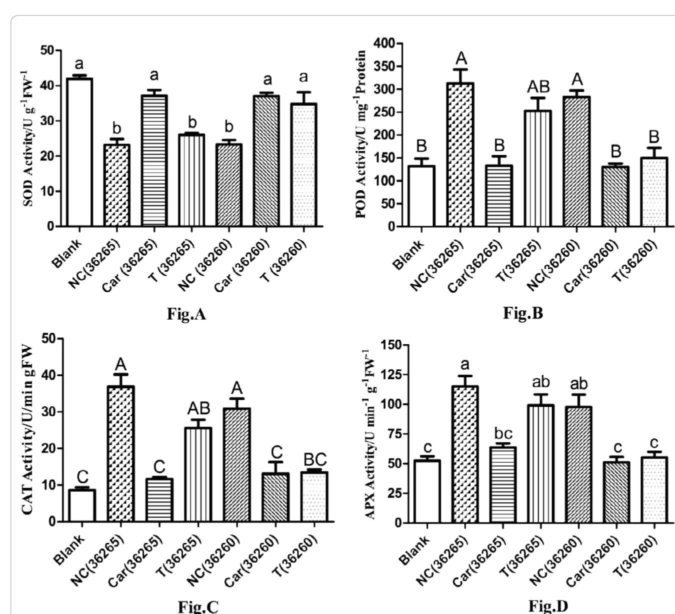
Structural analysis of metabolic active ingredients of XJAS-AB-11

Through extracting the fermentation broth of XJAS-AB-11, by isopyknic petroleum ether, normal butyl alcohol, chloroform, ethyl acetate, four brown yellow compounds were obtained, in weight of 2.717 g, 6.875 g, 28.354 g, 1.038 g respectively. Among which, the ethyl acetate extract showed the highest inhibition efficiency against all plant and animal pathogens, while the chloroform extract has no obvious effects. Hence, the ethyl acetate extract applied in the next experiment. Through TLC pre-test the chloroform/methanol (6:1) system chosen as a mobile phase (developing solvent) for the silica-gel column chromatography. As a final result for the silica-gel chromatography, two different monomers were obtained, which were XJAS-B (colorless acicular crystal) and XJAS-G (yellow powder).



Note: Fig A: SOD; Fig B: POD; Fig C: CAT; Fig D: APX. Blank (Blank group), NC (Negative control group), T (Treatment group), Car (Positive control group or Carbendazim treated group). (36265) and (36260) represents *Exserochilum turcicum* and *Bipolaris maydis*, respectively. Different capital letters and lowercases represents there has significant difference in total protein content of two groups (α=0.01) and (α=0.05), respectively.

Figure 3: Enzyme activity changes of enzymes after different treatment.



Note: Fig A: SOD; Fig B: POD; Fig C: CAT; Fig D: APX. Blank (Blank group), NC (Negative control group), T (Treatment group), Car (Positive control group or Carbendazim treated group). (36265) and (36260) represents *Exserochilum turcicum* and *Bipolaris maydis*, respectively. Different capital letters and lowercases represents there has significant difference in total protein content of two groups (α=0.01) and (α=0.05), respectively.

Figure 4: Enzyme activity changes of enzymes after different treatment.

According to ^{1}HNMR (Figures S7-S14), ^{13}CNMR (Figure S8 and Figure S11) and API-MS data set (Figure S6 and Figure S9), the monomer XJAS-B, with a molecular weight of 226.1, identified as Cyclo-(D-leucyl-trans-4-hydroxy-L-proline) [(3R,7R,8aS)-7-hydroxy-3-isobutyl hexahydro-pyrrolo[1,2-a] pyrazine-1,4-dione] (Figure 5), while XJAS-G, with a molecular weight of 302.0, identified as 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (Figure 6).

Naringenin-chalcone synthase (*CHS*) gene

A total of 823 bp and 1014 bp of XJAS-AB-11 *CHS* partial gene was amplified by Bsn and BpsA primers, respectively. Both sequence data showed high similarity with *Bacillus subtilis* BSn5 naringenin-chalcone synthase gene (99% and 97%, respectively). Based on the similarity and aligned gene length, the *CHS* partial sequence amplified by BpsA primer after TA cloning was selected for further analysis. Firstly, the selected *CHS* gene was translated into amino acid sequence with a total of 337 amino acids via ORF Finder system, and then the similar amino acid sequences were found through searching SWISS database. The phylogenetic tree (Figure S14) inferred from the amino acid sequence showed high homology with some experimentally verified prokaryotes and eukaryotes CHS amino acid sequence, such as those of *Streptomyces griseus* CHS, *Medicago sativa* CHS malonyl-CoA binding subunit, *Arachis hypogaea* stilbene synthase, and *Gerbera hybeid* CHS.

The secondary and three-dimensional structures of the *CHS* gene (Figure S12 and S13) showed the richness of α-helix and random coil structure in the XJAS-AB-11 *CHS* protein secondary structure. In addition, the spatial structure of this gene was highly conserved despite the considerable difference in the gene sequence between prokaryotes and higher plants, and their distant relationship. Moreover, high homology of *CHS* active site amino acid was also observed with *Arachis hypogaea* stilbene synthase, *Streptomyces griseus* CHS, and *Gerbera hybeid* CHS (Table 3).

Furthermore, the comparative analysis between XJAS-AB-11 CHS protein sequence and *Streptomyces griseus* CHS showed high homology between their amino acid sequences (Figure 7). Moreover, both of them also have similar amino acid sequences such as CMIYP and poly-G, which play a conclusive role in constructing of the spatial structure. Likewise, the catalytic site also contains the same amino acids, Cysteine (Cys) and Proline (Pro).

Discussion

Although *Bacillus subtilis* is a common bacteria, but not all of them possess the potentiality to inhibit the growth of plant/animal pathogens, as those isolated from medical plant *Alhagi pseudalhagi* Desv collected from Xinjiang. Despite the high inhibitory effect on different pathogens, these two bacteria, XJAS-AB-11 and XJAS-AB-13, were only effective on plant pathogens; this might be explained by one of these two reasons: (1) both of them could not produce any secondary metabolites that which is efficient on inhibiting animal pathogens; (2) they can produce some metabolites but not enough to observe the effects due to low production.

When plants are infected, they instantly produce and accumulate huge amount of reactive oxygen--called oxidative burst phenomenon; this is a most important early response, also one of the fastest response for initiating of defense mechanism against pathogens [27,28]. Thus, the activity changes of reactive oxygen related defense enzymes, SOD, POD, CAT, and APX, could be used as important parameters to indicate plant infection level. In this study, these defense enzymes showed increased activity, but those of SOD activity decreased. This is an interesting response, which needs further study to be explained.

The cyclic dipeptide XJAS-B isolated from XJAS-AB-11 is a derivative of cyclic dipeptide leucine-proline. Although there are several reports related to dipeptides isolated different organisms, for example, marine bacteria [29,30], however, no reports about finding of cyclic dipeptide from any medical plants or in any living bacterial strain. Thus, this is the first report about a cyclic dipeptide, (i.e., XJAS-B (cyclo-[D-leucyl-trans-4-hydroxy-L-proline])), isolated from a bacterial endophyte of medical plant *Alhagi pseudalhagi* Desv. In the other hand, XJAS-G is belonging to Quercetin. Quercetin and its derivatives are widely distributed among the plant world and it has been known to have anti-tumor, anti-inflammation, anti-platelet aggregation, anti-oxygen free radicals and vascular dilation properties [31]. The fermentation broth of XJAS-AB-11 showed inhibitory effect only on *Exserohilum turcicumand* and *Alternaria mali*, but showed no apparent effects on animal pathogens. Interestingly, the monomers XJAS-B and XJAS-G were only effective on controlling animal pathogens. This might be due to (1) other active compounds in the fermentation broth of XJAS-AB-11 worked together with the two

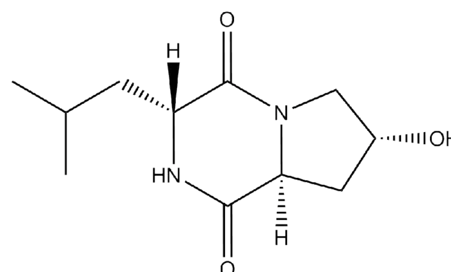


Figure 5: Chemical structure of compound XJAS-B.

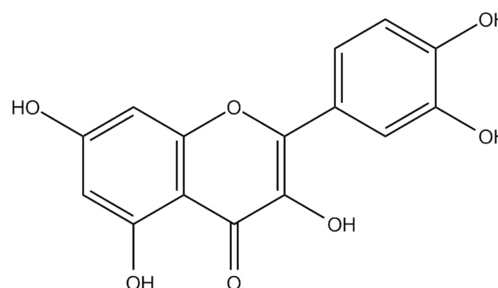


Figure 6: Chemical structure of compound XJAS-G.

	A	B	C	D	E	F	G	Substrate	Extender	Product
1	THN-RPPA	RNR	FF	CHN	CMIYP	TECCA	PGGLDGAGGGGGG	M-CoA	Mal	THNap
2	Stilbene	KRRA	FF	CHN	TLIGP	SETTS	PGGLDGPGGGGGA	pCoum	Mal	RESVR
3	Chalcone	KRKA	FF	CHN	TMIGP	SETTS	PGGLDGPGGGGGG	pCoum	Mal	Chalc

Note: A=protein name: THN-RPPA is *Streptomyces griseus* CHS; Stilbene is *Arachis hypogaea* Styrene synthetase; Chalcone is *Gerbera hybrid* CHS. B=CoA Tunnel Residues, C=Gatekeepers, D=Catalytic Triad, E=Cyclization Pocket, F=Coumaroyl Pocket, G=Geometry Shaping residues

Table 3: Comparison of *Streptomyces griseus* (1), *Arachis hypogaea* (2), *Gerbera hybrid* (3) type III polyketide synthase` active site amino acid sequences.

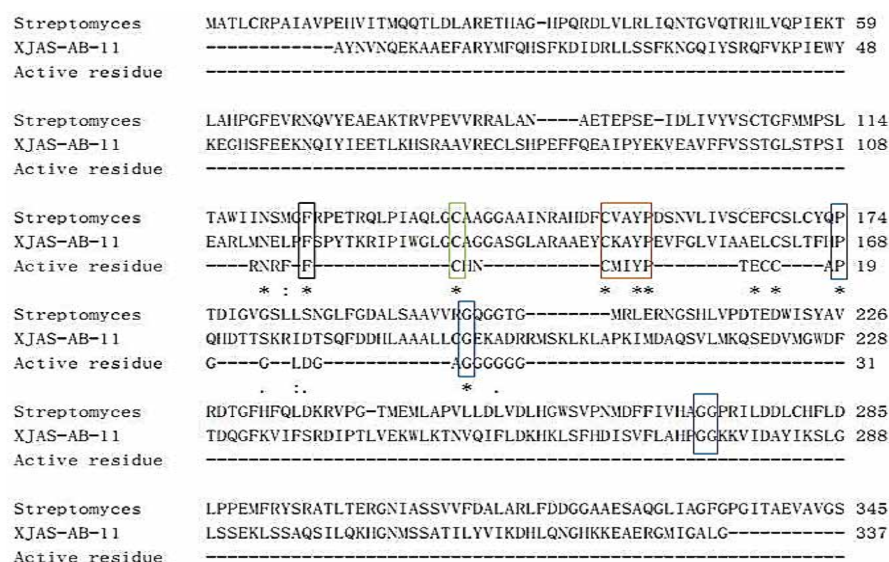


Figure 7: Cluatal W comparison of amino acid sequence of XJAS-AB-11 CHS and *Streptomyces griseus* CHS.

monomers, which becomes more potent against plant pathogens, and (2) fermentation dilutes the concentration of the monomer, making it less effective against animal pathogens.

In the last decade, as a result of the widespread use of PCR and DNA sequencing, 16S rDNA sequencing has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel bacteria in clinical microbiology laboratories. particularly important in the case of bacteria with unusual phenotypic profiles, rare bacteria, slow-growing bacteria, uncultivable bacteria and culture-negative infections. Not only has it provided insights into aetiologies of infectious disease, but it also helps clinicians in choosing antibiotics and in determining the duration of treatment and infection control procedures.

For bacterial identification, 16S rDNA sequencing has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel bacteria in clinical microbiology laboratories. Using 16S rDNA sequences, numerous bacterial genera and species have been reclassified and renamed, classification of uncultivable bacteria has been made possible, phylogenetic relationships have been determined, and the discovery and classification of novel bacterial species has been facilitated. In the last decade, sequencing of various bacterial genomes and comparison between genome and 16S rDNA gene phylogeny has confirmed the representativeness of the 16S rDNA gene in bacterial phylogeny [32-35].

Endophytes of medical plants could produce the same or similar metabolites with host plant. For example, plants in the Yew genus produce anticancer chemical, paclitaxel; similarly, endophytes of this plant were also able to produce the same chemical [36]; endophytes of Podophyllum produce podophyllotoxin [37], of Camptotheca, produce analogue of camptothecin [38], of Ginko produce flavonoids compounds [39]. This phenomenon may be due to horizontal transfer of genetic materials between host plant and its endophytes, which might be mutual exchange [14,40-42]; Prokaryotic cell genome sequencing pointed out that horizontal transfer of genetic materials is the most important mechanism of evolution [43-45]. Nevertheless, phylogenetic relationship between the species is always different despite the genetic material's transfer. During the horizontal gene transfer from eukaryotes to prokaryotes, gene(s) could undergo accelerated evolution and lost; as a result, the genetic relationship between the transferred gene(s) would be far. Moreover, the species misassignment caused by DNA contamination during gene transfer, may also phylogenetic relationships, even after the transfer of same genetic material [46].

In this study, the comparison result of *CHS* active site amino acid sequence showed that despite of differences of nucleic acid sequences in different species, the translated product (i.e., CHS amino acid sequence), is highly similar to those of *Actinomycetes*, as well as higher plant *Medicago*; thus, it is also possible that the endophytes of *Alhagi pseudalhagi* Desv, XJAS-AB-11, has its independent *CHS* gene that similar to higher plant.

In conclusion, the mechanism of gene horizontal transfer has been not clear, but this study further supported the probability of functional gene horizontal transfer between endophyte and its host plant. Moreover, this study provided useful data for development and utilization of new eco-friendly pesticide.

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Compliance with ethical standards

Conflict of Interest

All authors declare that they have no conflict of interest.

Ethical Approval

This article does not contain any study with human participants or animals performed by any of the authors.

References

1. FAOSTAT (2012) Statistical database of the food and agriculture of the united nations.
2. Kang ZS (2010) Current status and development strategy for research on plant fungal disease in China. Plant Protection 36: 9-12.
3. Yan MH (2004) The applied research of endophytic bacteria in biological control of plant disease. Biotechnol Infor 3: 8-10.
4. Shi JY, Chen WX, Liu AY (2006) Advance in the study of endophytes and their effects on control of plant disease. Acta Ecological Sinica 26: 2395-2401.

5. Liang JG, Zhu LH, Wu JA, Sang JL, Yao HL, et al. (2007) Control efficiency of biocontrol strain B-3 against pepper wilt and its identification. Acta Phytophylacica Sinica 34: 529-533.
6. Yi YJ, Xiao LT, Wang RZ, Bo LY, Liu EM, et al. (2007) Growth effect of endophyte B-001 (*Bacillus subtilis*) on tobacco seedling and its fluctuation. Acta Phytophylacica Sinica 34: 619-623.
7. Strobel GA (2003) Endophytes as sources of bioactive products. Microbes Infect 5: 535-544.
8. Bian JC, Feng YH, Yang J, Ding YW, Jing H (2010) Research Progress in Traditional Chinese Medicine Endophytes. GuangMing J Chinese Med (CJGMCM) 25: 164-165.
9. Bary A (1866) Morphologie und physiologie der pilze, flechten und myxomyceten. W. Engelmann.
10. Chen JH, Liu JJ, Zang GG, Li YJ, Zhuo LN (2004) Screening of Taxol-producing Endophytic Fungi and Regulation of Fermentation Condition. J Central South University (Sciences and Technology) 35: 65-69.
11. Zhang LQ, Guo B, Li HY, Zeng SR (2000) Preliminary study on the isolation of endophytic fungus of catharanthus roseus and its fermentation to produce products of therapeutic value. Chinese Traditional and Herbal Drugs 31: 805-807.
12. Stone JK, Bacon CW, White JF (2000) An overview of endophytic microbes: Endophytism defined. Microbial endophytes 3: 29-33.
13. Sturz AV, Christie BR, Nowak J (2000) Bacterial endophytes: Potential role in developing sustainable systems of crop production. Crc Cr Rev Plant Sci 19: 1-30.
14. Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by Taxomyces andreanae, an endophytic fungus of Pacific yew. Science 260: 214-216.
15. Strobel GA, Miller RV, Martinez-Miller C, Condron MM, Teplow DB, et al. (1999) Cryptocandin, a potent antimycotic from the endophytic fungus Cryptosporiopsis cf. quercina. Microbiology 145: 1919-1926.
16. Yang XW, Jiang YM, Li JS, Lou ZC (1996) A New Flavonal Glucoside from Aerial Parts of Manaplant Alhagi (Alhagi pseudoalhagi). Chinese Traditional and Herbal Drugs 27: 707-711.
17. Ailat S, Dilnur T, Ablimit A, Stiwaldi H (2005) Studies on Flavones Compounds from Alhagi pseudoalhagi. Natural Product Research and Development 17: 1-2.
18. Qian CR, Huang YX (2003) Laboratory Experiments in Microbiology. Beijing: Peking University Press p: 215.
19. Geris dos Santos RM, Rodrigues-Fo E, Caldas Rocha W, et al. (2003) Endophytic fungi from Melia azedarach. World J Microbiol Biotechnol 19: 767-770.
20. Conn VM, Franco CMM (2004) Analysis of the endophytic actinobacterial population in the roots of Wheat (*Triticum aestivum* L.) by terminal restriction fragment length polymorphism and sequencing of 16S rRNA Clones. Appl Environ Microbio 70: 1787-1794.
21. Li HY, Qing C, Zhang YL, Zhao ZW (2005) Screening for endophytic fungi with antitumor and antifungal activities from chinese medicinal plants. World J Microbiol Biotechnol 21: 1515-1519.
22. Buchanan RE, Gibbons NE (1984) Bergey's manual of determinative bacteriology. (18thedn.), Beijing: Science Press.
23. Dong XZ, Cai MY (2001) Common Bacterial Systems Identification Manual. Beijing: Science Press.
24. Chen JX, Wang XF (2006) Experimental Guide for Plant Physiology. (2ndedn.), Guangzhou: South China University of Technology Press.
25. Nakano C, Ozawa H, Akanuma G, Funa N, Horinouchi S (2009) Biosynthesis of aliphatic polyketides by type III polyketide synthase and methyltransferase in *Bacillus subtilis*. J Bacteriol 191: 4916-4923.
26. Aynazar Z, Ghopur M, Abdukadir A, Alimjan A, Gulzira Z (2012) Isolation of Endophytic Bacteria from Alhagi pseudalhagi Desv. and Analysis of Active Metabolites. Acta Botanica Boreali-Occidentalia Sinica 32: 1466-1473.
27. Song FM, Zheng Z, Ge XC (1996) Role of Active Oxygen and Membrane Lipid Peroxidation in Plant-Pathogen Interactions. Plant Physiol Commun 32: 377-385.
28. Wojtaszek P (1997) Oxidative burst: An early plant response to pathogen infection. Biochem J 322: 681-692.
29. Ai F, Xu QZ, Yang Y, Liu XY, Shi XQ, et al. (2006) Bioactive cyclodipeptides extracted from marine microbes in east China Sea. Academic Journal of Second Military Medical University 27: 22-24.
30. Yao Y, Tian L, Li J, Pei YH (2007) Cyclo-dipeptide Metabolites from The Broth of Marine Bacterium Bacillus sp. Chinese J Med Chem 17: 310.
31. Wang YF, Wang XH, Zhu YT (2003) Advancement of Researches in Quercetin. Nat Prod Res Dev 15: 171.
32. Fox GE, Magrum LJ, Balch WE, Wolfe RS, Woese CR (1977) Classification of methanogenic bacteria by 16S ribosomal RNA characterization. Proc Natl Acad Sci USA 74: 4537-4541.
33. Gupta R, Lanter JM, Woese CR (1983) Sequence of the 16S Ribosomal RNA from Halobacterium volcanii, an Archaebacterium. Science 221: 656-659.
34. Snel B, Bork P, Huynen MA (1999) Genome phylogeny based on gene content. Nat Genet 21: 108-110.
35. Woo PCY, Lau SKP, Teng JLL, Tse H, Yuen K (2008) Then and Now: Use of 16S rDNA Gene Sequencing for Bacterial Identification and Discovery of Novel Bacteria in Clinical Microbiology Laboratories. Clin Microbiol Infec 14: 908-934.
36. Wang JF, Lv HY, Su WJ (2000) Study on taxol production of plant endophytic fungi. Microbiology 27: 58-60.
37. Puri SC, Nazir A, Chawla R, Arora R, Riyaz-Ul-Hasan S, et al. (2006) The endophytic fungus Trametes hirsuta as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. J Biotechnol 122: 494-510.
38. Liu JH, Yu BY (2004) Isolation of endophytic fungi from camptotheca acuminala and the screening method of antitumor secondary metabolite produced by the fungi. J Plant Resou Env 13: 6-10.
39. Zhao QY, Fan MT, Shi JL (2007) Isolation and identification of flavones-forming endophytes from Ginkgo bilob L. Acta Agriculture Boreali-occidentalis Sinica 16: 169-173.
40. Mower JP, Stefanovic S, Young GJ, Palmer JD (2004) Plant genetics: Gene transfer from parasitic to host plants. Nature 432: 165-166.
41. Lawrence JG (2001) Catalyzing bacterial speciation: correlating lateral transfer with genetic headroom. Syst Biol 50: 479-496.
42. Boucher Y, Doolittle WF (2000) The role of lateral gene transfer in the evolution of isoprenoid biosynthesis pathways. Mol Microbiol 37: 703-716.
43. Nara T, Hshimoto T, Aoki T (2000) Evolutionary implications of the mosaic pyrimidine-biosynthetic pathway in eukaryotes. Gene 257: 209-222.
44. Ochman H (2001) Lateral and oblique gene transfer. Curr Opin Genet Dev 11: 616-619.
45. Dujon B, Sherman D, Fischer G, Durrens P, Casaregola S, et al. (2004) Genome evolution in yeasts. Nature 430: 35-44.
46. Hall C, Brachat S, Dietrich FS (2005) Contribution of horizontal gene transfer to the evolution of Saccharomyces cerevisiae. Eukaryot Cell 4: 1102-1115.