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Siderophore Producing by Endophytic Bacterial Strain YBS106 with Antifungal Activity against *Fusarium oxysporum*

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The aim of this study was to quantify the siderophores production of YBS106, isolated from *Arisaema erubescens* using improved sugar-aspartic acid (MSA) as a selective medium. The antifungal effect of this bacteria was then assessed against cotton wilt pathogen (*Fusarium oxysporum*) at varying concentrations of $FeCl_3$. This strain was identified using morphological, biochemical and physiological characteristics, 16S rDNA sequence homology and phylogenetic analysis. Strain YBS106 produced fluorescent siderophores in MSA medium, the relative content of which amounted to 83%. The siderophore produced by strain YBS106 exhibited significant inhibitory activity against *F. oxysporum* growth under low iron conditions. Strain YBS106 was identified as *Bacillus atrophaeus*.

Key words: Endophytic bacteria, Siderophore, *Fusarium oxysporum*, *Bacillus atrophaeus.*

Siderophores are low-molecular mass compounds (<1,500 Da) with high iron affinity (Neilands 1995) that allows soil microorganisms to sequester and solubilize ferric iron in iron poor environments. They can improve vegetal growth by increasing plant nutrient availability through iron uptake and preventing the growth of soil borne pathogens due to iron limitation (Chaiharn *et al*. 2009; Miethke and Marahiel 2007; Sayyed and Chincholkar 2009). Siderophores can be divided into two major categories, fluorescent and non-fluorescent, both of which can be quantitatively determined with the chrome azurol sulfonate (CAS) assay (Schwyn & Neilands, 1987).

Endophytes are a bacterial resource of great significance in biological control of pathogens (Cui *et al*., 2008) mediated through a number of mechanisms including competition, antifungal production, siderophore production and induction of plant systemic resistance. The previously identified endophytic bacterial strain, YBS106, has been shown to exhibit high antagonism against the cotton wilt pathogen, *Fusarium oxysporum*. Considering the potential ability of YBS106 in capacity to produce siderophores, the working hypothesis is that it can also exert biocontrol activity against *F. oxysporum* on cotton plants. Accordingly, the aim of this study was to quantify the siderophores production of previously characterized strains of YBS106.

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MATERIALS AND METHODS

Experimental materials

The endophytic bacteria YBS106 was isolated from the floral organ of *Arisaema erubescens* (Wall.) Schott.. *Escherichia coli* and *F. oxysporum* were supplied by the Key Laboratory of Special Biological Resources in Mountain Emei (Leshan Normal Universlty).

Detection of solid plate

Universal CAS blue detection solution and liquid MSA medium were prepared according to previously described methods(Schwyn & Neilands, 1987). Solid test plates were prepared by mixing MSA liquid medium with 5% CAS blue

detection solution and 2% agar. The endophytic bacteria, YBS106, was inoculated into LB liquid medium and activated for 12 h at 28°C 200 r/min to finally form a culture fluid. Activated YBS106 was streaked onto solid LB plates and cultured for 24 h at 28°C. Bacteria plugs (diameter, 8 mm) were formed and placed centrally in solid test plates prior to inverted cultivation at µ28°C for 2 da ys. Plates were then evaluated for the development of discoloration around the colony using a digital camera for general photography. In parallel, YBS106 culture fluids (10 µl) were titrated on filter papers (diameter, 8 mm) and cultured for 2 d. Fluorescence images were then recorded under long-wave UV radiation using a gel imaging system. *E.coli* was used as the negative control in this study.

Quantitative determination of siderophore

A previously described empirical approach was used for quantitative determination of siderophore(Schwyn & Neilands, 1987) with the following modifications: YBS106 cultures were prepared as a 1% (v/v) suspension in 30 ml MSA liquid medium and cultured for 24 h at 28°C, 200 rpm. Culture supernatant was obtained by centrifugation for 15 min at 10 000 rpm and debris removed by filtration (Millipore, diameter 0.22 µm). Samples were prepared by mixing the sterile filtrate with an equal volume of CAS blue detection solution and siderophore content was determined by measurement of OD680 (As) after an adequate response period at room temperature. Double-distilled water was used as the blank control and supernatant without the inoculating strain was used as the reference (Ar). The concentration of siderophore was expressed as siderophore units (Su) and calculated using the following equation: Su= [(Ar-As)/Ar] ×100% (Tang *et al*., 2013).

Inhibition of *F. oxysporum* by MSA culture supernatants

$FeCl_3$ was added to liquid MSA medium to generate solutions containing F_e^{3+} at 0, 10, 50 and 100 µmol/l. YBS106 culture suspension (1%) was added to liquid MSA medium and cultured for 24 h at 28°C, 200 rpm. Sterile supernatant was obtained by centrifugation for 15 min at 10 000 rpm followed by filtration. Liquid MSA medium without the inoculating strain was used as the control.

Solid potato dextrose agar (PDA) medium (1%) was dissolved in sterile supernatant, mixed and allowed to solidify. Controls plates were prepared using deionized water and 100 µmol/l FeCl3. A *F. oxysporum* fungus plug (diameter, 8 mm) was placed at the center of PDA plates (mycelium side down), and incubated at 25°C/90% humidity. Each experiment was performed in triplicates. The growth diameter of pathogenic fungi was measured after 3 days.

Endophyte identification

Bacterial morphology was observed by scanning electron microscopy after 24 h culture in beef extract peptone at 28°C. Identification of physiological and biochemical characteristics were examined as previously described(Dong & Cai, 2001) including features such as utilization of sole carbon and nitrogen sources, acid and alkali resistance, salt resistance, vopes-prokauer test, optimal growth temperature and pH value and enzyme (urease, oxidase, phenylalanine deaminase) activity.

16S rDNA sequences analysis was performed following PCR amplification using the following universal primers: 27F/1495r forward primer 5'-AGAGTTTGATCCTGGCTCAG-3'. 27F/1495r reverse primer: 5'-ACGGCTACCTTG TTACGACT-3', Total YBS106 DNA was used as template the template in a 50µl reaction volume. PCR amplification was performed using the following conditions: 94°C/5 min, 94°C/40 s, 62°C/40 s, 72°C/40 s, for 36 cycles followed by 72°C for 10 min. PCR products were sequenced (Sangon Biotech (Shanghai) Company Limited) and sequence homology was analyzed in GenBank. A phylogenetic tree was constructed using soft MEGA 4.0 (Molecular evolutionary genetics analysis).

RESULTS

Solid plate test

A more distinct color change and greater discoloration radius produced by YBS106 was observed in solid MSA test plates compared with negative control plates (Plate I, FigureA, Figure B). Color changes in this test result from siderophore chelation of iron and thus indicated siderophore production by YBS106 in MSA medium. Furthermore, the fluorescence detected around the YBS106 bacteria plug was greater than that detected around the negative control (Plate I, Figure C, Figure D), thus demonstrating that a

fluorescent siderophore was produced by strain YBS106 in MSA.

Quantitative siderophore determination

Following quantitative analysis, the siderophore activity of this YBS106 culture supernatant was calculated to be 83.0% according to the previously described formula. The As/Ar value of this sample (OD680) was 0.17, thus indicating a relatively high siderophore content.

Siderophore production capacity was classified according to the following system: + was assigned for each 0.2 decrease in As/Ar between 0 and 1.0 (Manjanatha *et al*., 1992). The siderophore secretion capacity of YBS106 was + + + + based on this classification system. The As/Ar of bacteria with high siderophore production capacity (+ + +) was generally less than 0.5 (Manjanatha *et al*., 1992), thus indicating that YBS106 is a strain with high siderophore production capacity.

Inhibition of *F. oxysporum* growth by MSA supernatants

Multiple comparisons of experimental data obtained following investigation of the effects of F_e^{3+} concentration on MSA supernatant inhibition of *F. oxysporum* were performed using DPS statistical analysis software(Tang, 2009). ANOVA ($P<0.01$) demonstrated that F_e^{3+} concentration significantly affected YBS106 supernatant inhibition of *F. oxysporum* growth. LSD multiple comparison tests showed no significant difference in colony diameters produced by the two control groups without YBS106 supernatant. However, significant differences were detected in the presence of Fe3+ at 0, 10 and 50 µmol/l (Table 1).

Table 1. Influence of YBS106 supernatant cultured with varying Fe^{3+} concs on *F. oxysporum* growth

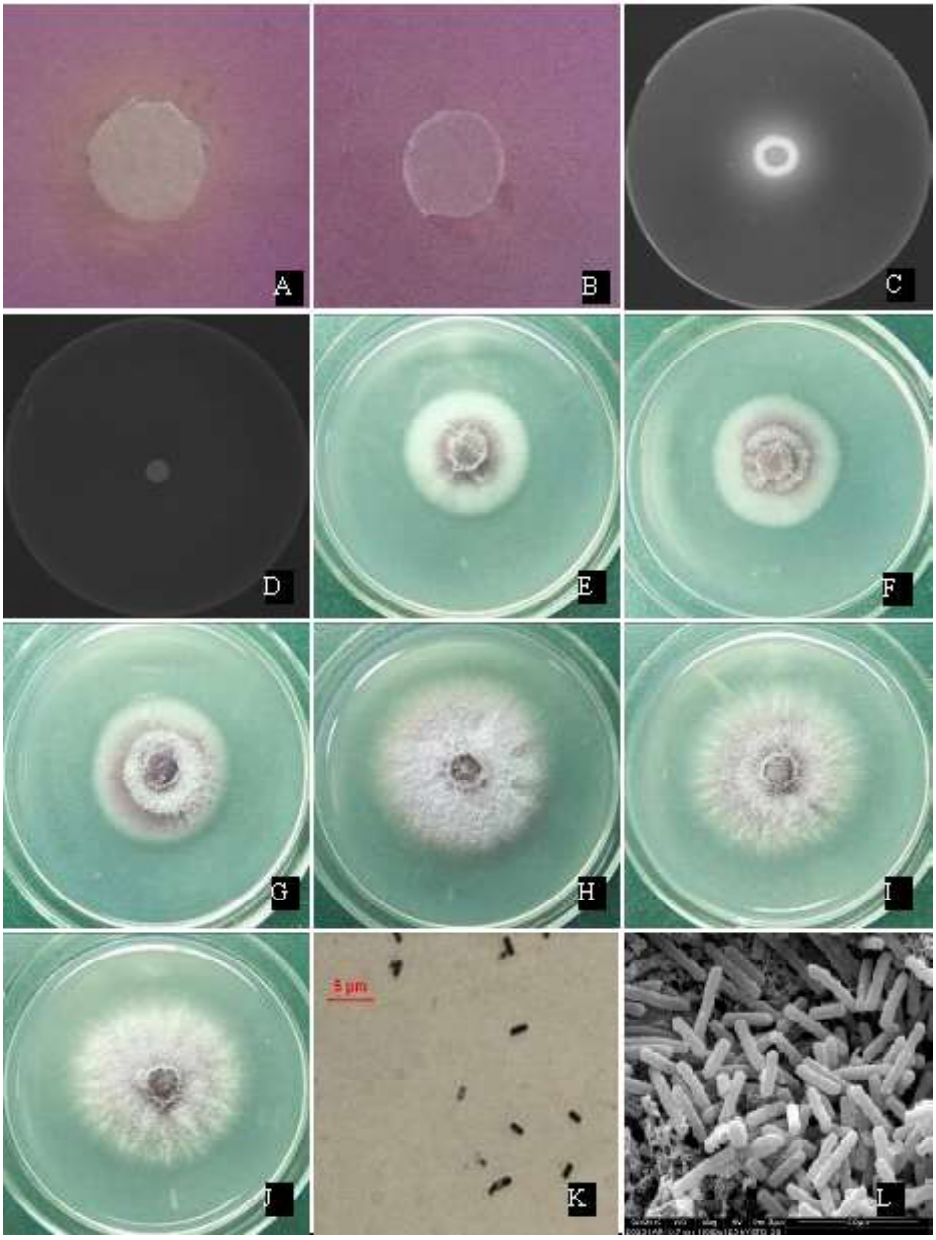
Fe^{3+} (µmol/l)	0	10	50	100	CK1	CK2
Coenobium diameter (cm)	3.60±0.06 cC	3.70±0.06 cC	3.97±0.03 bB	5.03±0.03 aA	5.10±0.03 aA	5.13±0.03 aA

Previous reports indicated that the amount of siderophores secreted by bacteria was reduced in the presence of increased concentrations of free Fe^{3+} in the surroundings (Chen *et al*., 2008). Inhibition of *F. oxysporum* growth in the presence of siderophore containing supernatants decreased with increased Fe^{3+} concentrations in MSA medium. It can be speculated that this phenomenon is due to the reduction in YBS106 siderophore secretion capacity at Fe^{3+} high concentrations (Plate 1, Figures E-J). Culture of *F. oxysporum* at 25°C/90% humidity showed no significant difference in mycelium diameters on PDA plates, although these were produced in the presence of varying F_e^{3+} concentrations (Plate I, Figures A and B), thus demonstrating that Fe^{3+} did not inhibit the growth of this pathogenic fungus. The smallest mycelial diameter was produced on PDA plates in the absence of F_e^{3+}, representing maximal inhibition (Plate I, Figures E-J), this occured at the highest siderophore concentration in all treatments. These data confirmed the observation that the siderophore secretion capacity, and consequently the inhibitory effect, of YBS106, on *F. oxysporum* growth correlated inversely with Fe^{3+} concentration.

Strain identification

YBS106 cultured for 24 h on beef extract peptone plate produced dry surfaced, opaque colonies with irregular edges containing a dark brown soluble pigment. Cells were Gram-positive, rod-shaped (Plate I, Figures K and L), spore-forming, singly or in pairs and no crystal.

Analysis of the physiological and biochemical characteristics of YBS106 revealed that this strain was able to reduce nitrate to nitrite, negative for oxidase, phenylalanine deaminase and lecithinase activity, positive for catalase and VP activity, negative for indole production and citrate utilization, positive for starch hydrolysis and able to utilize D-fructose, D-mannitol, trehalose, D-mannose and D-sorbitol as sole carbon sources. The optimum temperature for growth was in the range from 28°C to 30°C and although some growth was still apparent at 40°C, growth was completely



A. Normal image of YBS106 on solid detection plate.
B. Normal image of *E. coli* on solid detection plate.
C. Fluorescent image of YBS106 on solid detection plate.
D. Fluorescent image of *E. coli* on solid detection plate.
E. Inhibition of *Fusarium oxysporum* growth by YBS106 culture supernatant (0 μmol/l Fe^{3+}).
F. Inhibition of *Fusarium oxysporum* growth by YBS106 culture supernatant (10 μmol/l Fe^{3+}).
G. Inhibition of *Fusarium oxysporum* growth by YBS106 culture supernatant (50 μmol/l Fe^{3+}).
H. Inhibition of *Fusarium oxysporum* growth by YBS106 culture supernatant (100 μmol/l Fe^{3+}).
I. Inhibition of *Fusarium oxysporum* growth (100 μmol/l Fe^{3+}).
J. Inhibition of *Fusarium oxysporum* growth (deionized water).
K. Optical microscope view of YBS106 morphology (×1000).
L. Scanning electron microscope view of YBS106 morphology (×10000 magnification).

Plate 1. Diameter of all coenobium and filter papers (including blank control), 8 mm

inhibited at 41°C. The optimum pH for growth was in the range from 5.7 to 6.8 and the optimum NaCl concentration for growth was in the range from 2% to 10%.

16S rDNA was amplified for sequence analysis using YBS106 total DNA as a template with a common 27F/1495r primer. The YBS106 16S rDNA sequence (1452 bp) was submitted to GenBank (accession number: EU835573). Homologous sequences identified by BLAST analysis of the YBS106 16S rDNA sequence were used to construct a phylogenetic tree (Fig. 2). YBS106 exhibited 100% homology with the 16S rDNA sequences of Bacillus subtilis and Bacillus atrophaeus. Strain YBS106 was identified as Bacillus atrophaeus on the basis of these combined observations.

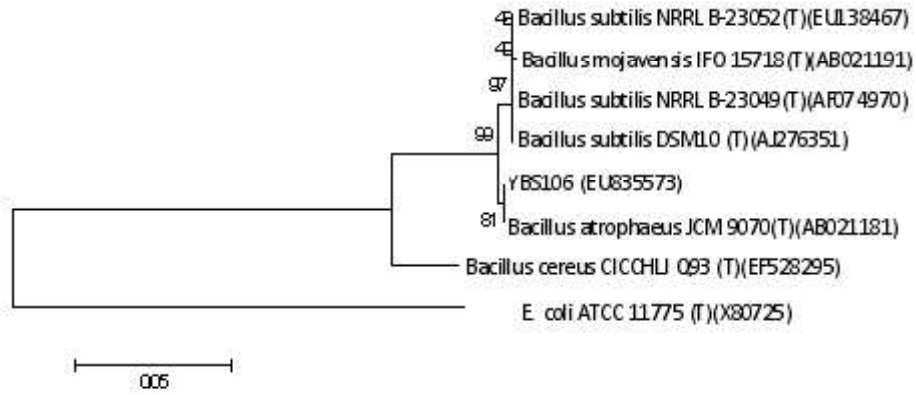


Fig. 2. Neighbor-joining phylogenetic tree based on 16S rDNA gene sequences of the strain YBS106 and related strains

DISCUSSION

Quantitative analysis of YBS106 siderophore production showed that the As/Ar of this strain was 0.17 at OD680, indicating a higher siderophore secretion capacity than other common strains. Furthermore, analysis of complex formation between Fe^{3+} and dye in samples (measured at 680 nm) demonstrated that complex formation was reduced in the presence of increasing siderophore concentrations indicated by lower As/Ar values.

Investigation of of *F. oxysporum* growth inhibition by YBS106 culture supernatants showed that YBS106 siderophores produced in low iron conditions (no additional Fe^{3+}) significantly inhibited *F.oxysporum* growth. However, no fungal growth inhibition was observed in rich iron conditions (100 µmol/l $FeCl_3$). These data indicated that, apart from siderophore production, no other antifungal activity (phenazine, rattan yellow pus streptozotocin, pyocyanin, 2,4 - diacetyl Garcinia phenol, nitrate pyrrole streptozotocin, Viscosinamide, HCN) was produced by YBS106 without induction in low-iron conditions (Haas & Defago, 2005).

Culture for 3 days on the PDA plates treated with the supernatant with varying Fe^{3+} concentrations resulted in generation small, but notable, differences in *F. oxysporum* coenobium diameter, indicating variations in the siderophore content of the supernatant added into medium. Higher siderophore concentrations were associated with greater inhibitory effects.

Siderophores can be divided into hydroxamate type, catechol type, carboxylic acid type and mixed type based on the chemical properties of chelating groups (Baakza *et al*., 2004). Structural type can be determined using the wavelength scanning method although pure siderophore samples are required for this analysis and more detailed structural characterization requires analysis using techniques such as liquid chromatography or gas chromatography. Lack of identification of the siderophore structure represents a limitation of this study that requires further investigation.

YBS106 was identified as *B. atrophaeus* by morphological, physiological and biochemical characterization and 16S rDNA sequence analysis. The majority of biocontrol bacteria with

siderophore production capacity are represented by *Pseudomonas* strains (Haas & Defago, 2005), while *Bacillus* strains are seldom reported. This study provides preliminarily confirmation that *Bacillus* has a high siderophore production capacity capable *F. oxysporum* growth inhibition.

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