JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2014. Vol. **8**(Spl. Edn. 1), p. 511-517

Isolation and Characterization of an Endophytic *Bacillus subtilis* Displaying Antibacterial and Anticancer Activities from *Dioscorea zingiberensis* C. H. Wright Rhizome

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(Received: 12 April 2014; accepted: 09 May 2014)

***Dioscorea zingiberensis* C. H. Wright is an important medicinal plant. We have firstly isolated several bacteria strains from the inner tissues of *D. zingiberensis* rhizome. One of them was named as SWB8 and identified as *Bacillus subtilis* via 16S rDNA analysis. Strain SWB8 is a Gram-positive spore-forming rod, aerobe or facultative aerobe with high salt and high alkali tolerance (pH 9.0, 9.0% NaCl), possessing a novel characteristic of the single chain fruiting body and produces several enzymes. The protein extract (PE) of strain SWB8 fermenting liquor showed inhibition effect against all six tested human pathogenic bacteria strains (*Staphylococcus aureus*; *Enterococcus faecalis*; *Escherichia coli*; *Salmonella typhi*; *Salmonella paratyphi* A; *Shigella dysenteriae*). The PE possessed significant anticancer activity against human pulmonary adenocarcinoma cells (A549) that the IC_{50} and IC_{90} values were 11.3 and 18.6 μg ml^{-1}, respectively. In contrast, human bone marrow mesenchymal stem cells (MSCs) showed high survivability with the IC_{50} value of 26.4μg ml^{-1}. *B. subtilis* SWB8 could be a potential source of antimicrobial agent or anticancer compounds with higher efficiency and lower toxicity.**

Key words: *Dioscorea zingiberensis* C. H. Wright, Endophyte, *Bacillus subtilis*, Activity.

Endophytes, the microorganisms, in the tissues of living plants without inflicting negative effects, have gradually become the important sources of novel natural products for exploitation in medicine, agriculture, and industry. Many endophytes make bioactive natural products that inhibit the growth of other organisms and synthesize the similar defensive natural products produced by the host[1,2]. Some endophytes produce phytohormones such as indole-3-acetic acid, cytokines, gibberellins and other plant growth-promoting substances[3]. There is nearly 300,000 plant species on the earth, and each individual plant is a host of one or more endophytes[1].

Dioscorea zingiberensis C. H. Wright, an important medicinal twining herb of the family *Dioscoreaceae*, mainly distribute in China. The rhizome of this plant contain with 45-50% starch, 40-50% cellulose and 1-5% saponin, and it is the major source of extracting diosgenin which is a steroidal sapogenin belonging to the triterpene group[4]. There is great interest of diosgenin in the pharmaceutical industry caused by the oestrogenic effect on the mammary gland[5,6], which can be used as steroid intermediate to synthesize steroid hormone such as cortical hormone, sex hormone and progestogen.

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Due to the increase in incidence of drug-resistant pathogen infections or the adverse effect of chemosynthesis drug, there is a resurgence of interest in finding alternates from endophytes of plants, especially the plants of medical value. The characteristics of the medicinal plant *D. zingiberensis* same absorb peoples to isolate valuable endophytic microorganisms and explore novel bioactive substance from endophytes. The study of endophytes of *D. zingiberensis* was just recently started, but some fungal endophytes have been isolated, including *Nectria*, *Fusarium*, *Rhizopycnis*, *Acremonium* and *Penicillium sp.*[7]. These fungi could provide some beneficial metabolites, such as beauvericin[8], saccharide elicitor[9], which played an important role in the process of inhibiting pathogens or enhancing *Diosgenin* production. All the endophytes of *D. zingiberensis* which have been reported belong to eumycophyta, but the endophytic bacteria remain largely unknown. In this study, we have firstly isolated endophytic bacteria from the inner tissues of rhizome of *D. zingiberensis* and systematically studied the biologic activities.

MATERIALS AND METHODS

Isolation of endophytic bacteria

The rhizome of *D. zingiberensis* which have been grown for two years were washed thoroughly under running tap water for 5 min, then subjected to 95% ethanol and followed by brief flaming to remove surface-associated microorganisms[10]. After that, the brown phloem of the rhizome was cut out, and then the remaining part was washed with sterile distilled water and soaked in 70% ethanol for 10 min. At last, the outer tissue was removed about 2-3 mm thick, and the inner tissue was made into 2-3 mm thick pieces and placed on the culture plates (1% peptone, 0.3% beef extract, 0.5% NaCl, 2% agar, 0.3% dry powder of *D. zingiberensis*, pH 7.2) which incubated at 32°C without antibiotics for several days. The newly formed colonies were inoculated to different isolation mediums to gain the desirable bacteria.

Morphological, biochemical and molecular characteristics of endophytic bacteria

The morphological characteristics of endophytic bacteria, including color, outline, elevation, translucency level, diameter of the colony, and size, shape, Gram type and arrangement of the endospores, were continuously recorded within 72 h. The VITEK 2 GP card (Biomerieux, France) was used to detect biochemical characteristics of the endophytic bacteria.

The bacterial isolate was grown in agitated liquid medium (0.3% beef extract, 1% peptone and 0.5% NaCl, pH7.2) at 32°C at 148 rpm for 48 h. The genomic DNA of the isolate was extracted to amplify 16S rDNA by using the established protocol[11], with minor modification. The primers used in the PCR were 27 f: (5' - GAGTTTGATCCTGGCTCAG-3') and 1492 r: (5' - CGGTTACCTTGTTACGACTT-3'). The 16S rDNA gene sequence of the bacterium was compared with available 16S rDNA gene sequences in GenBank databases using BLAST search facility at the National Center for Biotechnology Information (NCBI). The phylogenetic tree was constructed by using the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.

Acquisition, antibacterial and cytotoxic activities of protein extract

A colony (about 4-5 mm diameter) was inoculated to 250 ml Erlenmeyer flask containing 100 ml of liquid medium which incubated at 32°C at 146-148 rpm for 48-60 h. After centrifugation (5,000 rpm, 5 min), the supernatant was extracted twice with chloroform (50 ml, 30 min each time). The ivory white material isolated from the middle of two-phase was reserved. Both ethyl acetate and n-butyl alcohol was also used as extractant as mentioned before. After dried at room temperature, the ivory white material (IWM) was dissolved in double-distilled water. With the application of a saturated ammonium sulfate (SAS) concentration method, the certainly volumes of SAS and IWM solutions resulted in reaction mixtures of 40% ammonium sulfate at room temperature. At 4°C, the reaction mixture was set aside for 3 h and centrifuged (3,000 rpm, 10 min). The precipitated protein was dissolved in double-distilled water and dialyzed (3,500 MW-CO, Slide-A-Lyzer® Dialysis Cassette, Thermo) in 0.1 M phosphate-buffered saline (PBS, pH 7.2) to get rid of ammonium sulfate. After being dried at room temperature, the protein extract (PE) was dissolved in 0.1 M PBS, then sterilized (0.22 µm Millipore filter) and stored at -20°C.

With the disc diffusion method[12], the PE was used to determine the antibacterial activity

against six human pathogenic bacteria strains including *Staphylococcus aureus* (ATCC25923), *Enterococcus faecalis* (ATCC29212), *Escherichia coli* (ATCC25922) and clinical isolates of *Salmonella typhi*, *Salmonella paratyphi* A and *Shigella dysenteriae*. Each experiment was repeated for 5 times, and the diameter of the inhibition zone (mm) were presented in the form of mean ± standard deviations (SD).

Cytotoxic activity of the PE was evaluated by using MTT method[12,13]. Human pulmonary adenocarcinoma cells (A549) and human bone marrow mesenchymal stem cells (MSCs) were treated with PE at a final ratio of 7.2, 9.6, 12.0, 14.4, 16.8, 19.2, 21.6 and 24.0 μg ml^{-1}. The cell inhibition ratio was evaluated by absorbance value at 570 nm. All experiments were repeated for 4 times, and the data were presented in the form of mean ± SD. Both A549 and MSCs cells were treated with 24.0 μg ml^{-1} PE and incubated for 48 h to observe the morphologic features.

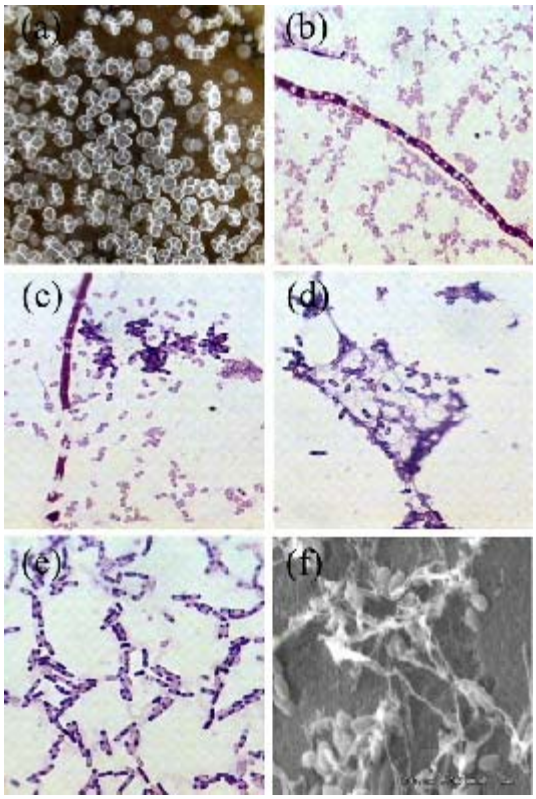

Fig. 1. Morphological features of the strain SWB8. (a) Colony on the solid medium which containing dry powder of *Dioscorea zingiberensis* C. H. Wright. (b) The single chain fruiting body, (c) the released endospores, (d) the shape of released endospores and (e) the single or double-arrangement bacteria (light microscopic observation). (f) The shape of released endospores (scanning electron microscopic observation).

RESULTS

Morphological and biochemical characteristics of strain SWB8

One of several bacterial strains isolated from the inner tissues of *D. zingiberensis* rhizome was named as SWB8. Strain SWB8 is an aerobe or facultative aerobe with growth temperature range from 10°C to 50°C (optimum temperature, 32°C), pH range from 6.0 to 9.0, and salinity range from 0.5-9.0% (NaCl). This strain produces α-amylase, α-glucosidase, α-galactosidase, β-1, 3-1,4-glucanase, and utilizes D-mannitol, D-maltose, D-glucose and amylopectin, and resists bacitracin. After 24 h post inoculation, the opaque rough reticulation bulge ivory colonies were observed, with the diameter about 3-4 mm (Fig.1a). The microscopic characteristics of this strain are as follows: gram positive, sporulation, single or double-arrangement, rod shape, about 1-1.5×3-5 μm (Fig.1e). At 48 h post inoculation, these ivory colonies gradually turned into yellowish, irregular and spread quickly. Furthermore, the spore filled fruiting bodies were observed as single long chains (Fig.1b). While the fruiting body was formed, the

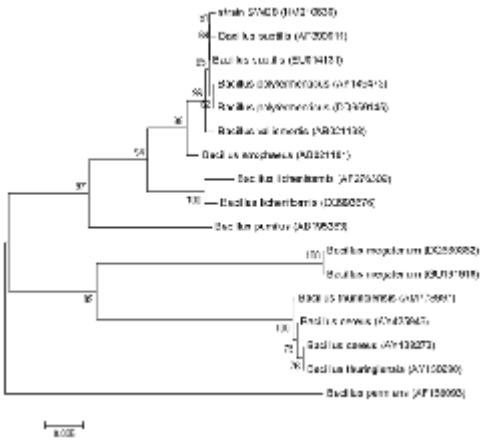

Fig. 2. The phylogenetic tree of strain SWB8 was constructed by using the neighbor-joining method, based on the 16S rDNA gene sequences. The numbers at the branches represent bootstrap values (1,000 bootstrap re-sampling). The GenBank accession number of each reference species is shown in parentheses

Table 1. The antibacterial activity of protein extract obtained from *B. subtilis* SWB8

Tested bacteria	Range (mm)	Mean (mm)	SD
Staphylococcus aureus (ATCC25923)	14-17	15.6	1.2
Enterococcus faecalis (ATCC29212)	20-22	21.3	0.8
Escherichia coli (ATCC25922)	16-20	18.0	1.1
Salmonella typhi	10-13	11.7	0.9
Salmonella paratyphi A	17-18	17.5	0.5
Shigella dysenteriae	8-10	9.0	0.9

The data represent diameter of the inhibition zones which were not recorded until the inhibition zone reached the maximum value. *Penicillin* G (60000 U) and *Gentamicin sulphate* (10 µg) were used as positive control. Positive standard: full transparent, uncolony and the diameter of inhibition zone larger than 6 mm.

oval colorless spores with the diameter 0.5-0.8×1-2 µm were released out from the long chains within 12 h (Fig.1c). In the initial stage, the released spores were adhered to each other (Fig.1d; f), but the phenomenon was disappeared soon. The spectacular scene was presented every 24 h since the fruiting body was formed. Based on the morphological and biochemical features, the strain SWB8 was definitely identified as genus *Bacillus*.

16S rDNA gene analysis of strain SWB8

The partial 16S rDNA gene sequence of strain SWB8 has been deposited in GenBank nucleotide sequences databases under the accession number HM210636. Phylogenetic tree was constructed based on the aligned 16S rDNA sequences of 17 isolates from soil, rhizosphere and internal tissues of plants. The 16S rDNA gene of the strain SWB8 shows the highest similarity to that of *Bacillus subtilis*. In the phylogenetic dendrogram, strain SWB8 forms the same clade with endophytic *Bacillus subtilis* AF399911 (Fig.2). Taking morphological and biochemical characteristics into consideration, the strain SWB8 was finally identified as *B. subtilis*.

Antibacterial and cytotoxic activity

The inhibition zone was presented on the plates which coated with test bacteria and reached the maximum value after 6-9 h incubation. The

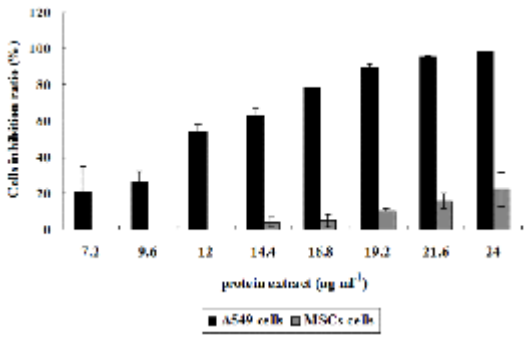

Fig. 3. Cell viability of A549 and MSCs cells that induced by the protein extract (PE). Both of A549 and MSCs cells were incubated with PE (7.2 µg ml^{-1}; 9.6 µg ml^{-1}; 12.0 µg ml^{-1}; 14.4 µg ml^{-1}; 16.8 µg ml^{-1}; 19.2 µg ml^{-1}; 21.6 µg ml^{-1}; 24.0 µg ml^{-1}) for 24 h. Inhibition ratio was evaluated by using MTT method. Black bars represent the inhibition ratio of treated A549 cells. Gray bars represent the inhibition ratio of treated MSCs cells. Error bars represent standard deviations.

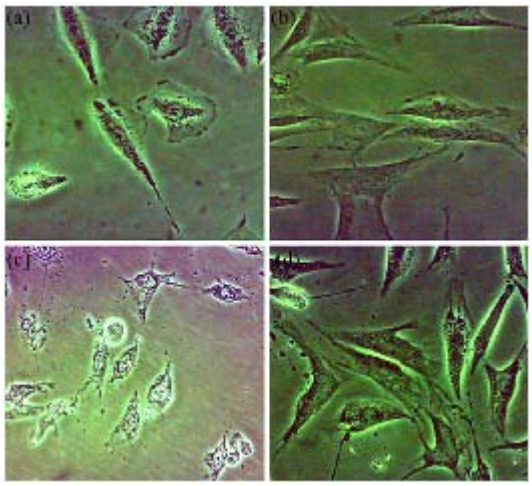

Fig. 4. The morphologic characterization of A549 and MSCs cells treated with the protein extract (PE). (a) A549 and (b) MSCs cells: negative control cells without PE. (c) A549 and (d) MSCs cells treated with PE (24.0 µg ml^{-1}). The morphologic features were recorded after 90 min incubation (inverted microscopic observation).

results (Table 1) show that all the tested pathogenic bacteria are inhibited by PE. *Enterococcus faecalis* (21.3±0.8 mm) and *Escherichia coli* (18.0±1.1 mm) are more sensitive to PE.

The results of MTT assay showed that the cytotoxicity of PE was selectively acted on A549 cells than MSCs cells (Fig.3). The inhibition ratio of A549 cells showed a positive correlation with the increasing concentrations of PE. The PE possessed significant anticancer activity against A549 cells that the IC_{50} and IC_{90} values were 11.3 and 18.6 μg ml^{-1}, respectively. In contrast, MSCs cells showed high survivability with the IC_{50} value of 26.4 μg ml^{-1}. During cytotoxic assay, only A549 cells showed distinguished morphologic changes (Fig.4c). Within 10 min, the adherent A549 cells started to shrink, and then the other morphological changes such as cytoplasmic blebbing, cytoplasm condensation were presented. After 90 min incubated with PE, the A549 cells were floated with the fragmented nucleus. In contrast, these morphological changes could not be observed in MSCs cells (Fig.4b; d).

DISCUSSION

Dioscorea zingiberensis C. H. Wright, an important medicinal plant for extracting oestrogen-like diosgenin has been proved with several endophytic fungi living in the inner tissues, including *Nectria, Fusarium, Rhizopycnis, Acremonium* and *Penicillium sp*.[7]. But it is never reported with endophytic bacteria. We firstly report that there are endophytic *B. subtilis* (strain SWB8) and other bacteria strains (unpublished data) exist in the inner tissues even the core area of the rhizome of this plant. We believe that the key factor for isolating endophytic bacteria is the modified isolation medium that added with dry powder of *D. zingiberensis*. Actually, these endophytic bacteria residing in the roots, stems, leaves of plants are known to have several dozen of genera members, such as *Enterobacter, Pseudomonas, Burkholderia, Arthrobacter, Sphingomonas* and *Bacillus*[14]. Previous studies indicate that one of the predominant strains in the tissues of plants is *Bacillus* bacteria, such as *B. megatherium, B. pumilus, B. cereus, B. licheniformis, B. subtilis* and other *Bacillus sp,* these endophytes show functionally benefit to the host plants for diseases[14-18]. Although the interaction relationship between *B. subtilis* strain SWB8 and *D. zingiberensis* is unclear, we suggested that the existence of *B. subtilis* SWB8 in the core area of the rhizome reflect the importance of this species for the host.

In natural settings, microorganisms most commonly exist as multicellular communities exhibiting a high degree of structure which have been described as biofilms. Soil *B. subtilis* have been proved that it is prone to form fruiting bodies under natural conditions, and its sporulation is tightly intertwined with the development of multicellular communities[19,20]. The fruiting bodies of *B. subtilis* SWB8 have been observed, but they appear as a bunch of single chains that are a little different from other reports. Besides, *B. subtilis* SWB8 is aerobe or facultative aerobe with high salt and high alkali tolerance that produces several enzymes and could utilize amylopectin. These characteristics might due to the adaptation of special living environment, the inner tissues of rhizome of *D. zingiberensis*. It is suggested that the difference between the internal environments of inner tissues of different plant might be a reason of biotype diversity.

Based on the extraction method, the active substances of *B. subtilis* SWB8 which could inhibit bacteria growth or induce cancer cell apoptosis are proteins or peptides that are the same as reported before[21-23]. The PE showed inhibition effect against all six tested human pathogenic strains that contained both Gram-positive and Gram-negative bacteria. It suggests that the PE might possess a broad spectrum antibacterial activity, and it is necessary that more pathogenic bacteria strains will be tested. We also found significant anticancer activity of PE against A549 cells. According to the morphological features, the cell proliferation inhibitory effect of PE is probably involved in the apoptosis despite we do not know either the main active compounds of PE or the mechanisms of cell death. The IC_{50} value of PE is higher than most of other anticancer agents[24], that it is probably attributed to the impure mixture. Interestingly, the PE does not display the same inhibition effect in MSCs cells which are multipotential progenitor cells capable of differentiating into multiple lineages of the mesenchyme that sensitive to regular anticancer agent, such as cyclophosphamide, 5-fluorouracil and taxol[25-27]. Perhaps, strain SWB8 could be a

potential source of antibacterial and anticancer compounds with higher efficiency and lower toxicity.

ACKNOWLEDGMENTS

This study was supported by grant nycytx-27-gw203 from the Ministry of Agriculture, P. R. China, and grant of Hubei 2011 Cooperative Innovation Center (grant 4), P. R. China.

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