INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17F–115/2018/20–4–745–749
DOI: 10.17957/IJAB/15.0551
http://www.fspublishers.org

Full Length Article

Isolation and Identification of *Viola philippica* Rhizosphere Bacteria and Analysis of their Fermentation Products

Li Wenjie[1†], Jiang Shuang[1†], Yin Xunzhe[1], Yu Xiuming[1], Guo Huanyu[1], Piao Xihang[1], Huang Jing[2] and Guo Yan[1*]
[1]*Changchun University of Chinese Medicine; Changchun, Jilin 130117, P.R. China*
[2]*First Hospital of Jilin University, Changchun, Jilin 130000, P.R. China*
[*]For correspondence: ccguoyan@163.com
[†]These authors contributed equally to this work

Abstract

Dilution plating procedure was adopted to isolate strains from the rhizosphere soil of medicinal plants *Viola philippica* (*Viola yedoensis* Makino). The pathogenic bacteria isolated in clinic were used as the indicator bacteria and the active strains were screened by fixed agar block method. Biological characteristics of the strains with a strong antibacterial activity were analyzed. The strains were identified and classified, and the antibacterial activity was determined by disk diffusion test. The antibacterial activity components were analyzed in combination with the extraction. Two strains Z1-7 and Z1-13a were screened from 21 strains, which showed a stronger antibacterial activity against the indicator bacteria. Z1-7 was an unknown species of *Streptomyces* and Z1-13a was California *Streptomyces*. The fermentation broth of the both strains had a strong antibacterial activity against *Staphylococcus aureus*, *Escherichia coli* and *Staphylococcus epidermidis.* The diameter of their inhibition zones was 10.0~15.5 mm. Z1-13a had a strong antibacterial activity against *Pseudomonas aeruginosa* and *Citrobacter freundii*. The antibacterial substances in Z1-7 and Z1-13a fermentation broth were lipid- and water-soluble substances, respectively. Both the above strains have a good antibacterial activity against common drug-resistant bacteria in clinic. Results suggest the potential value of strains Z1-7 and Z1-13a in research and development of new antibiotics against drug-resistant pathogens. © 2018 Friends Science Publishers

Keywords: *Viola philippica*; Rhizosphere bacteria; Isolation; Identification; Active ingredient

Introduction

The rhizosphere environment of medicinal plants is an important habitat for the development of new microbial resources. Studies have shown that many microorganisms isolated from the rhizosphere soil of medicinal plants have important biological activities (Thébault *et al*., 2010; Maria *et al*., 2011). More attention has been paid to the potential development and utilization value of medicinal plant rhizosphere bacteria as a microbial resource for screening new bioactive substances (Shaw *et al*., 2006).

Viola philippica (*Viola yedoensis* Makino) is one of the traditional Chinese medicinal materials. Pharmacological studies have confirmed good antibacterial, anti-inflammatory, antiviral and antitumor activities of *V. philippica* (Li *et al*., 2012; Oshima *et al*., 2013; Du *et al*., 2015; Zeng *et al*., 2016). In this study, strains that could antagonize activities of the pathogens were isolated and screened from the rhizosphere soil of *V. philippica.* These strains were identified and classified according to their biological characteristics. Active components in the fermentation broth of strains and their antibacterial activities were investigated at the same time, in order to develop and utilize the strains and their active components as a new microbial resource.

Materials and Methods

Materials

The test soil samples were collected from the root soil of *Viola philippic*. The test bacteria i.e., *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Citrobacter freundii* were provided by The First Affiliated Hospital of Jilin University. The test medium was modified Gauserime No. 1 medium, modified Gauserime No. 1 liquid culture medium, nutrient agar and nutrient broth.

Isolation, Purification and Preservation of Rhizosphere Bacteria

The soil sample was naturally air-dried and fully ground. Ten grams of the ground soil was dissolved in 100 mL distilled water, which was mixed and left standing fully, and then the soil suspension was diluted 10 times with sterile

distilled water (10^{-1}, 10^{-2} and 10^{-3}). The soil suspensions at these three concentrations were smeared evenly on the surface of the modified Gauserime No. 1 media with a sterile coated rod and inverted at 28°C for the culture for 7d. The strains with different colonial morphologies were selected and cultured on the modified Gauserime No. 1 media. The purified strains with single colony were picked and cultured on the tube slope of Gauserime No. 1 media for 7d, and then numbered and kept at 4°C in a refrigerator.

Screening of Active Strains

The isolated strains were streak-inoculated in four areas on the modified Gauserime No. 1 medium and inverted at 28°C for 7d for the culture, and then the single colonies were picked by agar block method for screening of antibacterial activity. *Staphylococcus aureus* and *Escherichia coli* were inoculated into test tubes containing nutrient broth and cultured at 37°C for 15 h. Turbidimetric method was used to adjust the concentration of bacteria solution to 1.5×10^6 CFU with normal saline, and then the solution was smeared on a nutrient agar plate. A single colony of the cultured strains was inverted on the plate and cultured at 37°C for 24 h. The diameter of inhibition zone was measured by cross method to screen the strains with the best antibacterial activity for the subsequent experiments.

Classification and Identification of Active Strains

The screened active strains were sent to Shanghai Meiji Bio-Pharmaceutical Technology Co. Ltd for 16S rDNA gene sequencing. The obtained sequences were checked in GenBank database for Blast retrieval. The 16S rDNA sequence of typical strains with a relatively high homology were selected as the reference objects, then CLUSTAL X software was used for multiple sequence alignment and computation of the sequence similarity between the test strains and the reference strains. The target sequence was aligned with DNA star software, and the phylogenetic tree between the test strains and the reference strains was constructed using MEGA 5 software.

Analysis on the Antibacterial Activity of Fermentation Broth of Active Strains

The activated mycelium block on the slant culture medium was inoculated on a fermentation medium for culture in a shake flask at 28°C at 150 $r\cdot min^{-1}$ for 7d. The culture medium was centrifuged at 4000 $r\cdot min^{-1}$ for 20 min, and supernatant was filtered through a 0.22 μm filter and the filtrate was stored in a sterile tube, which was the sterile fermentation broth. Five strains of the test target bacteria, *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Citrobacter freundii*, were cultured and smeared evenly on a nutrient agar plate after the concentration was adjusted according to the primary screening method described above. A sterile filter paper with 6 mm in diameter was soaked in the sterile fermentation broth, and then the sterile filter paper was taken out and dried in air in a sterile environment. The dried filter paper was affixed to the plate with indicator bacteria, cultured at 37°C for 24 h, and size of antibacterial circle was observed and recorded.

Analysis of Antibacterial Components in Fermentation Broth of Active Strains

Active fermentation broth was prepared as described above. The broth was separated into a butanol layer and a water layer after water saturation and butanol extraction. After evaporation at 37°C, two different phase solutions were respectively dissolved in the same volume of sterile water as that of the fermentation broth before extraction. The dissolved solutions were filtered by a 0.22 μm filter and filtrates were stored in sterile tubes. Antibacterial activities of the primary fermentation broth, the water layer fermentation broth and butanol layer fermentation broth of active strains were determined by disk diffusion method, and the antibacterial components were analyzed.

Results

Screening of Active Strains

Twenty one strains from the rhizosphere soil of *Viola philippica* were isolated according to differences in their culture characteristics. Screening results showed that nine of the 21 strains had antibacterial effects. The antibacterial activities of the two strains were, however, more obvious, which were named as Z1-7 and Z1-13a. It was found that the average diameter of inhibition zone of strain Z1-7 on *Staphylococcus aureus* and *Escherichia coli* was 12 mm and 16 mm, respectively, and that of strain Z1-13a it was 10 mm and 14 mm, respectively (Fig. 1).

Biological Characteristics of Active Strains

Strains Z1-7 and Z1-13a under light microscope (1000×) showed that the mycelia were fully developed and their sporangia were linear in shape, with positive Gram staining (Fig. 2a). It could be found by naked eye on the modified Gauserime No. 1 medium that both Z1-7 and Z1-13a colonies generated a lipid-soluble pigment, edge of colonies was regular, and surface was smooth and raised (Fig. 2b).

Classification and Identification of Active Strains

Results showed that the sequence similarity of strains Z1-7 and HZ01 (EU554550) was 99%, which could be an unknown species of *Streptomyces.* The sequence similarity of Z1-13a and NBRC 12750 (NR112257) was 99%,

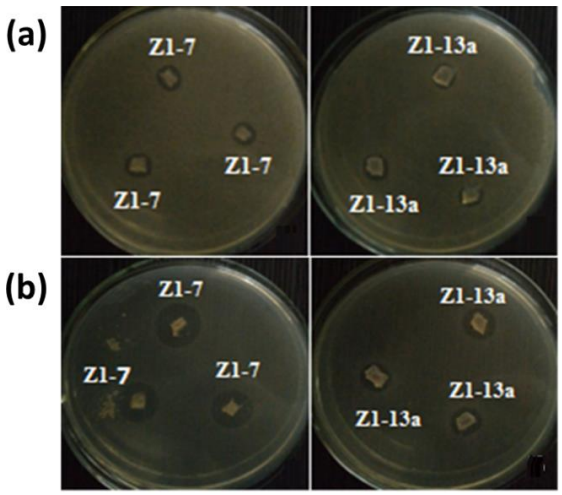

Fig. 1: Inhibition of Z1-7 and Z1-13a actinomycomes on the test *Staphylococcus aureus* (a) and *Escherichia coli* (b)

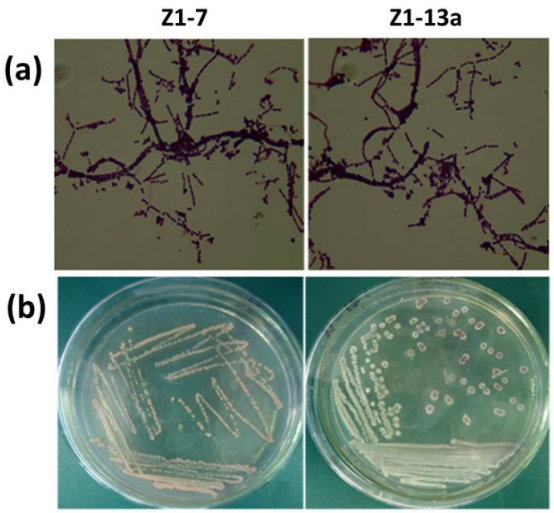

Fig. 2: Biological characteristics of strains Z1-7 s and Z1-13a
Under an ordinary light microscope; (b) Colony morphology

which should be California Streptomyces. However, exact taxonomic status of strains Z1-7 and Z1-13a needs further analysis (Fig. 3).

Analysis of Antibacterial Activity of Active Strain Fermentation Broth

Fermentation broth of the two strains Z1-7 and Z1-13a had a certain antibacterial effect on *Staphylococcus aureus*, *Escherichia coli* and *Staphylococcus epidermidis*, and the diameter of inhibition zone was 12.0~15.5 mm. In addition, Z1-13a had a strong antibacterial effect on *Pseudomonas aeruginosa* and *Citrobacter freundii*, and the diameter of their inhibition zone was 17.5 mm and 11.5 mm, respectively (Fig. 4).

Analysis of Antibacterial Components in Fermentation Broth of Active Strains

Metabolites in the water layer of strain Z1-7 fermentation broth by butanol extraction had no antibacterial activity. The metabolites in the butanol layer, however, had similar antibacterial activity as that of strain Z1-7, suggesting that the polarity of antibacterial substances from strain Z1-7 should be smaller, and the antibacterial substances could not be carbohydrates and proteins, but should be some flavonoids or other small polar substances. The metabolites in the butanol layer of Strain Z1-13a by butanol extraction had no antibacterial activity; whereas, in water layer it was same as that of strain Z1-13a fermentation broth, suggesting that the polarity of antibacterial substances from strain Z1-13a should be larger, which could be some carbohydrates or proteins (Fig. 5).

Discussion

Since the discovery of penicillin, antibiotics have been successfully used for the treatment of infectious diseases caused by microorganisms (Fsadni *et al*., 2015; Dekker *et al*., 2017). However, with wide use of antibiotics, bacterial resistance is serious (Bardoloi and Yogeesha Babu, 2017). Microbial metabolites are important source of bioactive substances in drug discovery (Kino *et al*., 2014; Morita and Nishino, 2017). Actinomycete is an important microorganism in rhizosphere environment that plays an important role in promoting the growth of plants and protecting plant roots from the invasion of pathogenic fungi. It has been reported that actinomycetes isolated from the rhizosphere of several plants, such as ginseng and *Arabidopsis*, has strong activities against various pathogenic bacteria (Ryu *et al*., 2003; Zhao *et al*., 2012; Zhang *et al*., 2013).

Studies based on the analysis of gene 16s rDNA by molecular biology in recent years have showed that the structure of rhizosphere microorganism community is significantly different from that of non-rhizosphere microorganism community. The influence of plant species on rhizosphere microorganism communities is the most obvious in contrast to that of other factors (Monciardini *et al*., 2002; You, 2005; Li and Liu, 2006). The rhizosphere environment of medicinal plants is different from that of other plants. The actinomycetes growing in the rhizosphere can interact with the medicinal plants for a long time to establish a mutualistic relationship, and some actinomycetes growing in such an environment may participate in some physiological metabolic processes of medicinal plants to produce some plant metabolic analogues or new active compounds (Butler *et al*., 2003; Delgado *et al*., 2011). The results from the classification of phylogenetic tree constructed based on 16S rDNA showed that the strain Z1-7 was an unknown species of *Streptomyces* and Z1-13a was California *Streptomyces*.

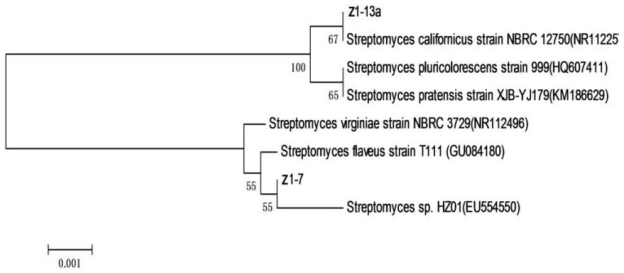

Fig. 3: Analysis of the strain phylogenetic tree constructed based on the nucleotide sequence of 16S rDNA

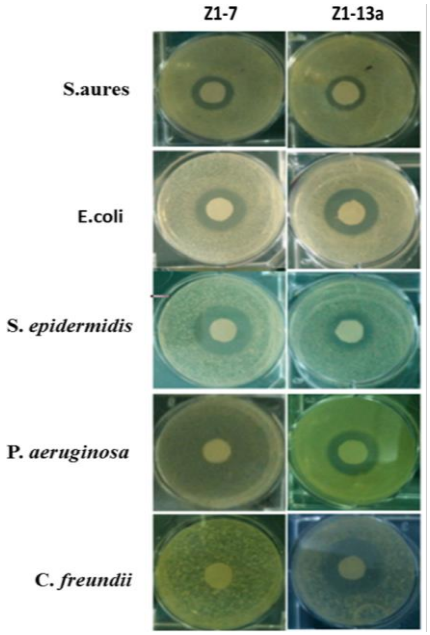

Fig. 4: Inhibition of the fermentation broth of active strains Z1-7 and Z1-13a on the test pathogens

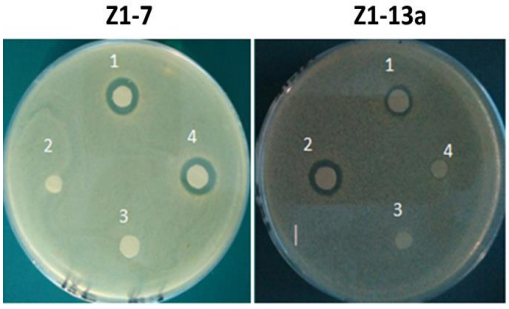

Fig. 5: Inhibition of active strains Z1-7 and Z1-13a fermentation broth after extraction on *S.aureus*
1 Fermentation broth; 2 Water layer; 3 Blank control; 4 Butanol layer

The fermentation broth of the two active strains had a strong antibacterial activity against *Staphylococcus aureus*, *Escherichia coli* and *Staphylococcus epidermidis.* Strain Z1-13a in addition had a strong antibacterial activity against *Pseudomonas aeruginosa* and *Citrobacter freundii* as well. The antibacterial components in fermentation broth were lipid- and water-soluble. In view of the current findings, studies need to be focused on large-scale fermentation of strains Z1-7 and Z1-13a to further clarify the components of antimicrobial substances and their chemical structures.

Conclusion

Two strains Z1-7 (unknown species of genus *Streptomyces*) and Z1-13a (California *Streptomyces*) were found to have good antibacterial activity against the common drug-resistant bacteria suggesting these strains promising for new drug discovery.

Acknowledgement

This study was funded by Scientific Research Program of Jilin Provincial Health and Family Planning (2014Z099), "12th Five-Year" Science and Technology Research Project of Education Department of Jilin Province (JIJIAOKE Union [2015] No. 333), Natural Science Founds of Science and Technology Department of Jilin Province (20160101224JC), Jilin Province College Students' Innovation and Entrepreneurship Training program (201610199135), and "13th Five-Year" Industrialization Cultivation Planning Project of Education Department of Jilin Province (JJKH20170739KJ).

References

Bardoloi, V. and K.V. Yogeesha Babu, 2017. Comparative study of isolates from community-acquired and catheter-associated urinary tract infections with reference to biofilm-producing property, antibiotic sensitivity and multi-drug resistance. *J. Med. Microbiol.*, 66: 927−936

Butler, J.L., M.A. Williams, P.J. Bottomley and D.D. Myrold, 2003. Microbial community dynamics associated with rhizosphere carbon flow. *Appl. Environ. Microbiol*., 69: 6793−6800

Dekker, A.R., T.J.M. Verheij and A.W. Velden, 2017. Antibiotic management of children with infectious diseases in Dutch Primary Care. *Fam. Pract.*, 34: 169−174

Delgado, S., F. Nunez, B. Sanchez, E. Bermudez and J.M. Rodriguez, 2011. Toxigenic microorganisms in medicinal plants used for ritual protection of infants. *Food Res. Int.*, 44: 304−309

Du, D., Z. Cheng and D. Chen, 2015. Anti-complement sesquiterpenes from *Viola yedoensis*. *Fitoterapia*, 101: 73−79

Fsadni, C., P. Fsadni, S. Fava and S. Montefort, 2015. Association of prevalence of rhinitis, atopic eczema, rhinoconjunctivitis and wheezing with mortality from infectious diseases and with antibiotic susceptibility at a country level. *Asia Pac. Allergy*, 5: 145−155

Kino, K., Y. Kotanaka, T. Arai and M. Yagasaki, 2014. A novel L-amino acid ligase from Bacillus subtilis NBRC3134, a microorganism producing peptide-antibiotic rhizocticin. *Biosci. Biotechnol. Biochem.*, 73: 901−907

Li, W., J.Y. Xie, H. Li, Y.Y. Zhang, J. Cao and Z.H. Cheng, 2012. *Viola yedoensis* liposoluble fraction ameliorates lipopolysaccharide-induced acute lung injury in mice. *Amer. J. Chin. Med.*, 40: 1007−1018

Li, Z.Y. and Y. Liu, 2006. Marine sponge Craniella austrialiensis-associated bacterial diversity revelation based on 16S rDNA library and biologically active Actinomycetes screening, phylogenetic analysis. *Lett. Appl. Microbiol.*, 43: 410−416

Maria, S., A.R. Rivelli and M. Kuffner, 2011. Interactions between accumulation of trace elements and macronutrients in *Salix caprea* after inoculation with rhizosphere microorganisms. *Chemosphere*, 84: 1256−1261

Monciardini, P., M. Sosio, L. Cavaletti, C. Chiocchini and S. Donadio, 2002. New PCR primers for the selective amplification of 16S rDNA from different groups of actinomycetes. *FEMS Microbiol. Ecol*., 42: 419−429

Morita, Y. and K. Nishino, 2017. Frontier of Pharmaceutical Microbiology: To Combat Multidrug-resistant Bacterial Pathogens. *Yakugaku Zasshi*, 137: 371−372

Oshima, N., Y. Narukawa, T. Takeda and F. Kiuchi, 2013. Collagenase inhibitors from *Viola yedoensis*. *J. Nat. Med.*, 67: 240−245

Ryu, C.M., M.A. Farag, C.H. Hu, M.S. Reddy, H.X. Wei and P.W. Paré, 2003. Bacterial volatiles promote growth in Arabidopsis. *Proc. Natl. Acad. Sci. USA*, 100: 4927−4932

Shaw, L.J., P. Morris and J.E. Hooker, 2006. Perception and modification of plant flavonoid signals by rhizosphere microorganisms. *Environ. Microbiol*., 8: 1867−1880

Thébault, A., B. Frey, E.A.D. Mitchell and A. Buttler, 2010. Species-specific effects of polyploidisation and plant traits of *Centaurea maculosa* and *Senecio inaequidens* on rhizosphere microorganisms. *Oecologia*, 163: 1011−1020

You, S.J., 2005. The microorganism community in an aerobic denitrification membrane bioreactor by 16S rDNA based molecular biotechnology. *Environ. Technol*., 26: 767−772

Zeng, H.R., B. Wang, Z. Zhao, Q. Zhang, M.Y. Liang and Y.Q. Yao, 2016. Effects of *Viola yedoensis*, makino anti-itching compound on degranulation and cytokine generation in rbl-2h3 mast cells. *J. Ethnopharmacol*., 189: 132−138

Zhang, H.Y., Q.H. Xue, G.H. Shen and D.S. Wang, 2013. Effects of actinomycetes agent on ginseng growth and rhizosphere soil microflora. *Chin. J. Appl. Ecol*., 24: 2287−2293

Zhao, K., P. Penttinen, Q. Chen, T. Guan, K. Lindström and X. Ao, 2012. The rhizospheres of traditional medicinal plants in Panxi, China, host a diverse selection of actinobacteria with antimicrobial properties. *Appl. Microbiol. Biotechnol*., 94: 1321−1335

(Received 05 October 2017; Accepted 20 November 2017)