

Identification and crude protein extract of endophytic bacteria strain KLXD06 antagonistic against MRSA isolated from *Hemsleya sinensis*

Mei Tang¹, Mingfu Gong^{1*}, Chenghao Xu¹, Sanlin Wu¹ and Fang Liu¹

¹School of Life Sciences, Leshan Normal University, Leshan, Sichuan, China

Abstract: *Hemsleya sinensis* Cogn has a variety of medicinal value. Strain KLXD06 is an endophytic bacteria isolated from *H. sinensis* exhibited significant inhibitory activity against methicillin-resistant *Staphylococcus aureus* (MRSA). This strain was identified by methods including 16S rDNA sequence homology and phylogenetic analysis morphological, biochemical and physiological characteristics analysis. Crude protein from KLXD06 was extracted by ammonium sulfate salting-out. The results showed that strain KLXD06 was identified as *Serratia marcescens*. Antibacterial crude protein from KLXD06 was extracted by ammonium sulfate salting-out, has a thermal stability.

Keywords: *Hemsleya sinensis* Cogn, Endophytic bacteria, methicillin-resistant *Staphylococcus aureus*, Identification, Antagonist

INTRODUCTION

Hemsleya sinensis Cogn belongs to *Hemsleya*, Cucurbitaceae, which contains a variety of active ingredients, and has a variety of pharmacological effects, including anti-tumor (Tao *et al.* 1999), broad-spectrum antibacterial (Li *et al.* 2005), antiviral (Tian *et al.* 2008), protect the liver (Shi *et al.* 1996), the treatment of gastrointestinal disorders (Liu *et al.* 2010), anti-inflammatory antitussive (Cheng *et al.* 2009) and so on, are widely used in clinical (Yang & Wang 2003).

Methicillin-resistant *Staphylococcus aureus* (MRSA) as a nosocomial infection multidrug resistant pathogens (Hiramatsu *et al.* 2002). All countries have the reports about MRSA. MRSA isolation rate of Chinese major hospitals is very high (Liu *et al.* 2013). MRSA have the ability of resistance a variety of clinical antibiotic including methicillin. Therefore, to solve the problem of drug resistance of MRSA, it has become a very important task to develop new anti-MRSA drugs.

In recent years, Strain KLXD06 screened from the endophytic bacteria of *H. sinensis* has strong activity of anti-MRSA. Purpose of this paper is to identify this strain by methods including 16S rDNA sequence homology and phylogenetic analysis morphological, biochemical and physiological characteristics analysis and to extract crude protein from KLXD06 antagonistic against MRSA by ammonium sulfate salting-out.

MATERIALS AND METHODS

Strains in this test

The endophytic bacteria strain KLXD06 was gained by isolating method with the massive root of *H. sinensis*.

*Corresponding author: e-mail: gongmingfu98@163.com

MRSA used in this test were gained from microbiological laboratory in Leshan Normal University.

Identification of strain KLXD06

Scanning electron microscopy was used to observe bacterial morphology after 24 h culture in beef extract peptone at 28 degrees centigrade. The method described by Dong & Cai (2001) was used for Identification of physiological and biochemical characteristics including features.

With universal primers: 27F/1495r, 16S rDNA sequences analysis was performed. Total KLXD06 DNA was used as template in a 50 μ L reaction volume for PCR amplification under the following conditions: 94 degrees centigrade/5 min, 36 cycles (94 degrees centigrade/40 s, 62 degrees centigrade/40 s, 72 degrees centigrade/40 s) followed, 72 degrees centigrade for 10 min. PCR products were sequenced and sequence homology was analyzed. With soft Molecular evolutionary genetics analysis (The version number was four) (Tamura *et al.* 2007), the phylogenetic tree was constructed.

Antibacterial protein extraction and antibacterial activity determination

Antibacterial protein extraction

The strain KLXD06 was inoculated into 100 mL medium of NB broth (Peptone 10.0g/L, beef extract 5.0g/L, Sodium chloride 5.0 g/L) and incubated at 28 degrees centigrade for 72-96 h with shaking at 160 rpm. The culture was centrifuged for 15 min under 5000 rpm, filtered by 0.22 μ m membrane filter. The filtrate was slowly added saturated ammonium sulfate solution to a saturation of about 60 percentage to stand overnight and centrifuged (12000 rpm, 10 min), the supernatant discarded, with 0.1M/L phosphate buffer (pH 6.8) to dissolve the precipitate, dissolving liquid was putted to minus 20 degrees centigrade to save backup.

Determination of Anti-MRSA activity

In the surface of Nutrient agar culture media (added agar 15.0 g/L in NB) plate, Four small holes with diameter of 7 mm were pressed. An aliquot of 0.1mL MRSA culture (approx. $10^9 \sim 10^{10}$ cfu mL^{-1}) was spread onto the culture medium surface. An aliquot of 100 μL 20 percentage, 60 percentage and 100 percentage concentration respectively of the crude protein solution was poured into three of the four holes. An aliquot of 100 μL sterilized distilled water was poured into another hole as the control. Triplicate for every treatment was set and incubated under 28 degrees centigrade for 48 h. The anti-MRSA activity of the crude protein solution was indicated by colony inhibited size (CIS), which was defined as the value of the radius of the crude protein solution minus the radius of the hole.

Determination of thermal stability of antibacterial crude protein

Crude protein extract lysate was put into water at 40 degrees centigrade, 60 degrees centigrade, 80 degrees centigrade, 100 degrees centigrade water bath for 15 min and water at 121 degrees centigrade water bath for 30 min. according to the aforementioned method for measuring the colony inhibited size, with without heat treatment the stock solution as control. Calculation of residual antibacterial activity (RAA). RAA was expressed as (CIS in treatment/ CIS in control) \times 100 percentage.

RESULTS

Strain identification

Colonies of Strain KLXD06 cultured on NA plate for 24 h were red, smooth, opaque, neat edges, slightly convex. Cells were Gram-negative, short rod, no capsule, no spores (fig. 1, fig. 2).

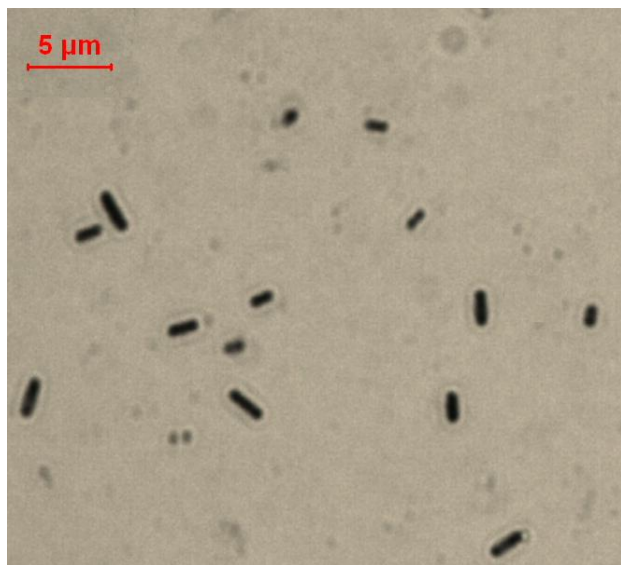


Fig. 1: Optical microscope view of KLXD06 morphology ($\times 1000$)

The strain KLXD06 in a nutrient broth medium was turbid diffusion. Significantly positive reaction were including in anaerobic growth, fermentation of glucose oxidase, catalase, VP measured, gelatin liquefaction, nitrate reduction, lysine decarboxylase, ONPG measured using citric acid. Significantly negative reaction were including arginine dual hydrolase trials; phenylalanine deaminase, urease, hydrogen sulfide, indole, methyl red (MR), using experimental malonate. This strain can used mannitol, xylose, sucrose, trehalose, mannose, maltose, D-sorbitol in maltose Sugar alcohols fermentation test. The optimum grow condition for growth was pH in the range from 5.7 to 6.8 and NaCl concentration in the range from 2 percentage to 10 percentage.

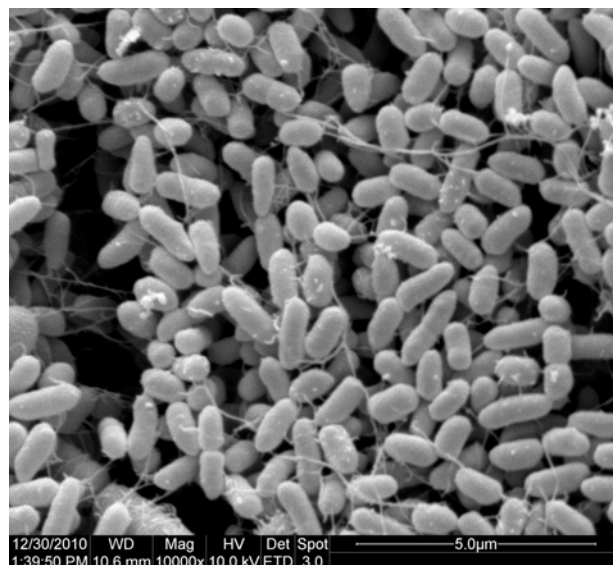


Fig. 2: Scanning electron microscope view of KLXD06 morphology ($\times 10000$ magnification).

16S rDNA sequence (1450 bp) of KLXD06 was submitted to Gen Bank. BLAST analysis was used for homologous sequences of the KLXD06 identified by 16S rDNA sequence. A phylogenetic tree was constructed by Homologous sequences (fig. 3). The 16S rDNA sequences of KLXD06 exhibited 100 percentage homology with of *Serratia marcescens* strain DAP29 (type strain, accession number in Gen Bank: EU302854). Based on these combined observations, strain KLXD06 was identified as *S. marcescens*.

Antagonistic activity against MRSA of crude protein from KLXD06

Antibacterial crude protein from KLXD06 had significant activity against MRSA (see table 1). Antagonistic effect of 100 percentage concentration of crude protein extracts against MRSA was more obvious, inhibition zone diameter reached 12 mm or more (see fig. 4). KLXD06 had the potential as a biological anti-bacteria.

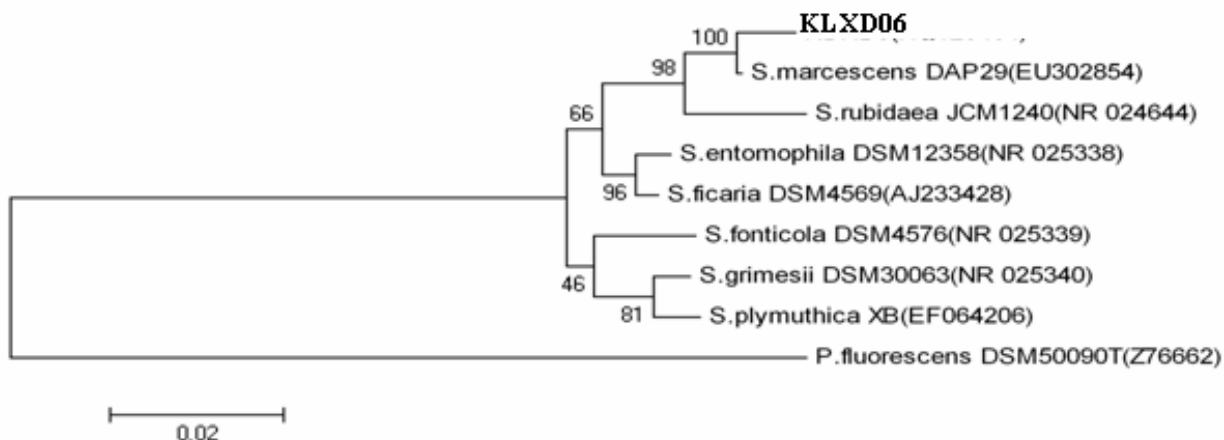


Fig. 3: Neighbor-joining phylogenetic tree of the strain KLXD06 with homologous sequences strains based on 16S rDNA gene sequences.

Note: The figures on the branches indicate the reliabilities. Gen Bank accession numbers are in the parenthesis. The boldfaces are strains for 16S rRNA sequencing. The scale bar represents 2 substitutions per 100 nucleotide position.

Table 1: Antagonistic activity against MRSA of different concentrations of crude protein extracts from strain KLXD06 isolated from *Hemsleya sinesis*

Different concentrations of crude protein extracts from KLXD06 (percentage)	Colony inhibited size of crude protein extracts from KLXD06 (mm)			
	1	2	3	Average
0 (Control)	0	0	0	0
20	11.6	10.2	11.4	11.1 aA
60	11.7	11.2	11.5	11.5 bAB
100	12.5	13.2	14.3	13.3 bB

Note: Same letters presented behind the data means no significant difference among tested isolates while different letters means the difference was significant.

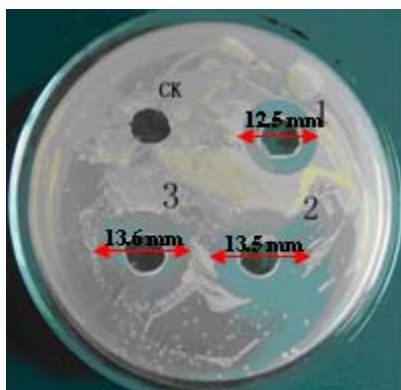


Fig. 4: Antagonistic activity against MRSA of 100% concentrations of crude protein extracts from strain KLXD06 isolated from *Hemsleya sinesis* Thermal stability of crude protein extract from KLXD06

Determination results of thermal stability of crude protein extract showed that crude protein from strain KLXD06 remained antibacterial activity against MRSA with increasing temperature (see fig. 5). Water Bath for 15 min, the residual antimicrobial activity at 40 degrees

centigrade and 60 degrees centigrade is consistent with the control group, remains 100 percentage antibacterial activity, the residual antimicrobial activity at 80 degrees centigrade and 100 degrees centigrade averaged 75.0 has been reported that many strains of anti-MRSA have been 121 degrees centigrade, the average residual antimicrobial activity was 35.5 percentage. The results showed that crude protein extract from KLXD06 had a thermal stability and can withstand temperatures below 121 degrees centigrade.

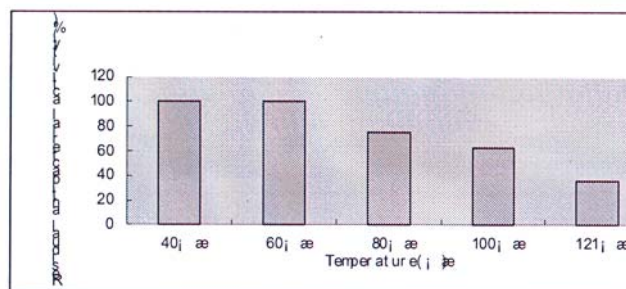


Fig. 5: Effect of temperature on the antibacterial activity of crude protein from endophytic bacteria strain KLXD06 isolated from *Hemsleya sinesis*.

DISCUSSION

KLXD06 was identified as *S. marcescens* based on 16S rDNA sequence analysis and morphological, physiological and biochemical characterization analysis. At present, anti-MRSA substances were screened *in vitro* antibacterial test from herbs and their extracts in China, which extracts of Scutellaria, berberine and other exhibited good antibacterial effect (Yang *et al.* 2006, Zuo *et al.* 2006). But the complexity of herbal ingredients and its suppression mechanism of bacteria more difficult. It has been reported that many strains of anti-MRSA have been screened (Liu *et al.* 2007, Huang *et al.* 2004). *S. marcescens* strain KDNB6 isolated from *Sophora alopecuroides* in Xinjiang, which had a conspicuous antagonistic effect on MRSA (Zhang *et al.* 2011). The other anti-MRSA strains screened from medicinal plants were also rarely reported.

Different concentrations of crude protein extracts from KLXD06 had antibacterial activity against on MRSA. It indicated that KLXD06 produced antimicrobial substances, had the potential to produce anti-MRSA drugs.

Crude protein extract from KLXD06 had a thermal stability, but what that is, how the nature, whether there are other antibacterial substances, anti-bacterial substances conditions and other issues arising from the need to be further explored.

ACKNOWLEDGMENTS

The authors thank to Leshan Normal University. Mingfu Gong is recipient of Leshan Normal University productivity grants (Grant No. Z1160).

REFERENCES

- Chen XJ, Wu YY, Kuang WJ, Miao SK and Zhou LM (2009). Experimental research on effect of Hemsleya tablets on anti-inflammatory and anti-tussive. *JSPS*, **31**(4): 153-154.
- Dong XZ and Cai MY (2001). Determinative manual of familiar bacteria system. Beijing Science Press, pp. 162-171.
- Hiramatsu K, Katayama Y, Yuzawa H and Ito T (2002). Molecular genetics of methicillin-resistant *Staphylococcus aureus*. *IJMM*, **292**(2): 67-74.
- Huang HQ, Lu JS, Zhang KS and Bao SX (2004). Characterization and phylogenetic analysis of a marine actinomycete with inhibitory effect on methicillin-resistant *Staphylococcus aureus* (MRSA). *CJMD*, **23**(6): 23-26.
- Li L, Zhu L, Liu R, Xie F, Zhao YY, Ma, XY and Zhou LM (2005). Hemsleya choline pharmacodynamic tests.

- SJPS*, **27**(3): 135.
- Liu J, Xie XL and Niu TG (2007). Screening and identification for Bacillus of having inhibitory effect against MRSA. *JCAU*, **12**(6): 20-23.
- Liu Y, Ma FL and Qiao H (2013). Investigation and analysis of the prevalence rate of nosocomial infection. *CJD*, **2013**(3): 236-237.
- Liu YL, Li XR, Fan JY, Xu QM and YANG SL (2010). Experimental research on effects of Xue Dan Wei chang pill on gastric ulcer. *Lishizhen Med. Mater. Med. Res.*, **21**(2): 301-302.
- Shi YQ, Yang PQ, Tai YM and Zhang WJ (1996). determination of content of Hemsleya A in the different fruits of Hemsleya a dolichocarp by RP-HPLC. *Tra. Chin. Med.*, **1996**(5): 20-21.
- Tamura K, Dudley J, Nei M and Kumar S (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *MBE*, **24**: 1596-1599.
- Tao CY, Yi YH, Lin HW, Xu QZ and Lu TS (1999). Study on anti-tumor activity constituents of *Hemsleya* root. *Acad. J. Second. Mil. Med. Univ.*, **20**(5): 70-72.
- Tian RR, Chen JC, Zhang GH, Qiu MH, Wang YH, Du L, Shen X, Liu NF and Zhen YT (2008). Hemsleya vitamin A and B, *in vitro* anti- HIV-1 activity. *Chin. Nat. Med.*, **6** (3): 214 -218.
- Yang MW, Lu F, Xu LJ, Liu YJ and Chen R (2006). Inhibition effects of 20 Heat-clearing and Toxin-resolving Chinese medical herbs on MRSA *in vitro*. *Chin. Pharm.*, **9**(2): 141-142.
- Yang Q and Wang XY (2003). Research progress of Chinese medicine hemsleya and its preparation. *Chin. Pharm.*, **9**(6): 76 -77.
- Zhang SC, Han S, Huang XY, Mei WL, Lin TX and Gong MF (2011). Identification of endophytic bacteria KDNB6 from *sophora alopecuroides* and anti-bacterial substance against MRSA. *Microb. Chin.*, **38**(6): 860-864.
- Zuo GY, Wang GC, Xu GL, Yu W, Peng H and Kuang WX (2006). Screen of anti-MRSA activity from 30 Chinese herbage medicine extracts. *Chin. J. Mod. Appl. Pharm.*, **23**(4): 293-294.