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Full Length Research Paper

Identification of a bacterium that produced an anti-mycobacterium tuberculosis activity

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The colonial morphology, the cells and the spores of a bacterium had been observed that could produce an anti-*M*ycobacterium *tuberculosis* antibiotic. By physiological and biochemical characteristics and by 16S rDNA analysis, this bacterium was identified as *Bacillus subtilis*.

Key words: Bacterium, identification, 16S rDNA, *Bacillus subtilis.*

INTRODUCTION

Natural products have played a major role in antibiotic discovery since 1941 when penicillin was introduced to the market. Currently, natural products are again the most important source for promising new antibiotic, and efforts have refocused on finding new antibiotics from old sources and new sources (Clardy et al., 2006; Luzhetskyy et al., 2007). In the course of screening the bacteria that could antagonize *Xanthomonas oryzae pv.oryzae* (a pathogen of rice), we had isolated a bacterium from the egg plant leaf in the suburb of Hangzhou City, Zhejiang Province, P.R. China. This bacterium could produce a novel antibiotic named BS. In previous paper, we had reported that BS has a potent antagonistic activity against *M. tuberculosi*, even if the *M.tuberculosis* was multidrug-resistant tuberculosis or MDR-TB (Chen and Yue, 2010). With BS's anti-*M.tuberculosis* activity being promising and with the aim to further research BS-producing bacterium as well as its antibiotic, it is of great value to investigate the classification position of the bacterium. The current approach used for identifying bacterial species is based on phenotypic and genomic properties (Pontes et al., 2007). In the case of bacteria with unusual phenotypic profiles, 16S rDNA sequencing is particularly important (Woo et al., 2008).

MATERIALS AND METHODS

Culture medium and microorganism

The liquid KMB (Kings Medium B Agar) culture medium: BBI company (peptone) 20 g, glycerol 15 ml, K_2HPO 1.5 g, $MgSO_4$, 0.75 g. The volume was adjusted to 1000 ml by distilled water. It was sterilized at 121℃ for 20 min. The KMB agar plate contained 15 g agar in 1.0 liter liquid KMB culture medium.

Microorganisms

BS-producing bacterium to be identified was isolated from the egg plant leaf in the suburb of Hangzhou City, Zhejiang Province, P.R. China and it was deposited in the Institute of Biotechnology, Zhejiang University, P.R. China.

The observation of the bacterium

A colony of BS-producing bacterium was incubated in liquid KMB culture medium at 37℃ until the AD600 reached 1.5. For the observation of colonial morphology, the bacterium culture was diluted 1×10^9 times, and was then poured on KMB agar plates. After 24 h of incubation at 37℃, colonies appeared on the plates. For the observation of the cells of the bacteria and for the detection of spores, the culture isolates were stained with Wirtz-Conklin spore staining as described in reference (Hamouda et al., 2002).

The major physiological and biochemical characteristics of BS-producing Bacterium

All the experiments relevant to the major physiological and biochemical characteristics, including Gram staining, V.P. test, starch hydrolysis ability test, litmus milk test, catalase test, isinglass hydrolysis test, casein hydrolysis test, glucose oxidative fermentation, low-temperature and high-temperature culture, nitrate reduction test and acid producing test were carried out as described in references (George et al., 2004; Chinese Academy of Sciences, 1978).

The analysis of 16S rDNA

The 16S rDNA sequence was PCR amplified using universal

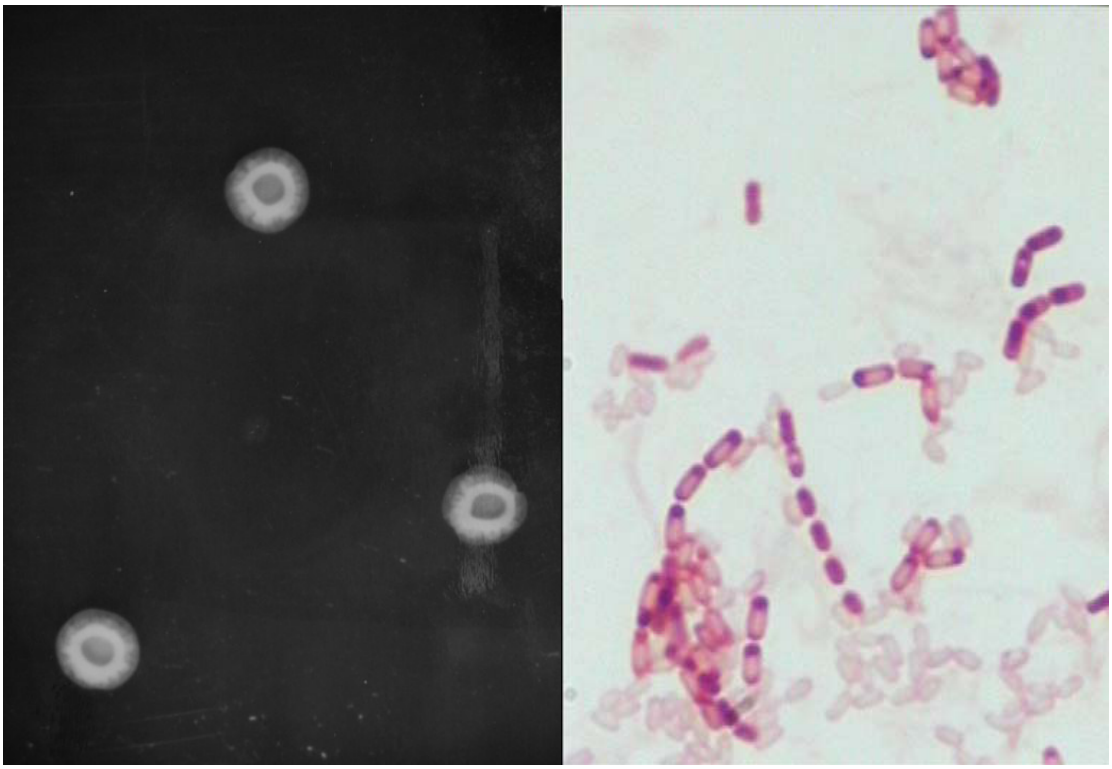

Figure 1. The BS-producing bacterium. Left: the colonial morphology of BS-producing bacterium. Right: the shape of BS-producing bacterium with Wirtz-Conklin spore staining being applied. Arrows pointed to the spores that had been removed from the sporangia.

bacterial primers as described in Weisburg et al. (1991). The standard cycling conditions were as follows: i) an initial denaturation at 94℃ for 5 min; ii) 30 cycles of denaturation at 94℃ for 30 s, annealing at 54℃ for 45 s and elongation at 72℃ for 45 s; iii) A final 10 min extension period at 72℃. Following visualization by electrophoresis on a 1% (w/v) agarose gel, PCR fragments were purified using the GFX PCR DNA purification kit (Amersham Bioscience, Swedish). Ligation was done with T4 DNA ligase (Fermentas, Canada), using pUCm-T vector, and it was transformed into *Escherichia coli* competent cells and small scale plasmid isolation using the Gene JETTM plasmid Miniprep Kit (Fermentas) according to the manufacturer's instruction. Selected clones identified as carrying the plasmid-borne insert of interest were sequenced by Shanghai Boya Bioengineering Technologies Corporation, P. R. China. The obtained sequence was aligned with the reference sequence retrieved from Genebank (NCBI) following BLAST searches and the sequence homology analysis was performed using DNAuser.

RESULTS

The colonial morphology and the shape of BS-producing bacterium

The colonial morphology of BS-producing bacterium is shown in Figure 1. Since the bacterium secreted a layer of special exopolysaccharide that covered the bacterium, judging by the colonial morphology only, no initial judgment could be made as to the type of bacterium. By Wirtz-Conklin spore staining, it was observed that the bacterium was a spore-forming rod and it had sporangia with an oval spore inside as has been shown in Figure 1.

The major physiological and biochemical characteristics of BS-producing bacterium

The major physiological and biochemical characteristics of BS-producing Bacterium are given in Table 1. From Table 1, it could be seen that all the analyzed characterization of the bacterium was identical to those which had been described as a standard *Bacillus subtilis* in the reference. Based on the physiological and biochemical characteristics described above, the bacterium was tentatively classified as a member of *B. subtilis*.

The analysis of 16S rDNA

The PCR fragment of 16S rDNA of BS-producing bacterium and the sequence of the 16S rDNA are respectively shown in Figures 2 and 3. The sequence homology analysis shows that BS-producing bacterium shared 99 to 100% similarity with the model *B. subtilis*. In other words, there were only two base pair differences

Table 1. The major physiological and biochemical characteristics of the newly isolated bacterium.

Biochemical test		Test bacterium	Standard *B. subtilis*	Features reaction		Test bacterium	Standard *B. subtilis*
Gram stram		+	+	Low temperature test	5℃	—	—
V. P. trial		+	+		10℃	+	+
$NO_3^- \longrightarrow NO_2^-$		+	+		15℃	+	+
Restore the litmus milk		+	+				
Contact enzyme		+	+	High temperature test	45℃	+	+
					50℃	+	+
					55℃	—	—
Mensurate of glucose oxidase fermentation		**Acid produced by fermentation**	**Acid produced by fermentation**	Oxygen demand		+	+
Produce acid	Glucose	+	+	Hydrolyze	Starch	+	+
	Arabinose	+	+		Glutin	+	+
	Xylose	+	+		Casein	+	+
	Mannitose	+	+				
				Lateral flagella		+	+

between BS-producing bacterium 16S rDNA and the model *B. subtilis* 16S rDNA.

DISCUSSION

In 1943, an American named Selman Waksman, together with his co-workers, discovered that a fungus called *Streptomyces griseus* produced an antibiotic substance which they named "streptomycin". Streptomycin was the first antibiotic used against *M. tuberculosis* (Zetterstrom, 2007). As had been reported in our previous paper, a bacterium, which was newly isolated by us, produced a novel and potent anti-*M. tuberculosis* antibiotic. This paper had successfully identified that BS-producing bacterium was a member of *B. subtilis*. This genus comprehends a heterogeneous group of Gram-positive, aerobic or facultative anaerobic, endospore-forming bacteria (Fritze, 2004). Although there were reports that some *B. subtilis* strains could produce bioactivesubstance (Magali et al., 2008; Gilardi et al., 2008; Ryu, 2007), it had not been documented that a *B. sublilis* strain could produce a potent anti-*M. tuberculosis* substance. In the face of the global resurgence of TB and the rapid emergence of MDR-TB (Coleman et al., 2001; Lourenco, et al, 2008; Biava et al., 2008), our previous success in the determination of the MICs for BS against TB and our present success in the identification of BS-producing bacterium were undoubtedly of significance. Our future research work should focus on the evaluation of BS's anti-*M. tuberculosis* activity *in vivo,* which is more important but which is also more challenging due to the fact that at this time we have a difficulty in obtaining sufficient BS that is needed to conduct

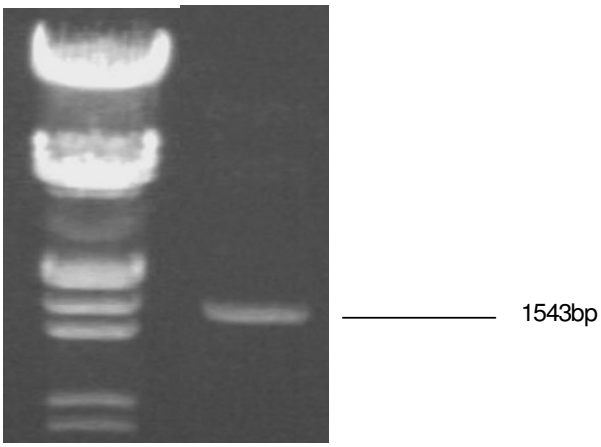

Figure 2. PCR amplification of 16S rDNA.

ATGGGCGGCCGCCTGCAGACCAGGTCTAGAGTTTGATCATGGCTCAGGACGAACGCTGGC

GGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCG

GCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAAC

CGGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGG

CTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA

GGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCC

CAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAG

CAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAAC

AAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAAAAAGCCACGGCTAACT

ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTA

AAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGG

TCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGG

TGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACT

GACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGgTAGTCCACGCC

GTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCAT

TAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGT

CCCGCACAAGCGGTGGAGCATGTGGTTTATTCGAAGCAACGCGAGAACCTTTACCAGGTC

TTGACATCCTCTGACAATCCTAGAGAATAGGACGTCCCCTTCGGGGGCAGAGTGAACAAG

TGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC

GCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGAC

AAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAC

Figure 3. The sequence of 16S rDNA of the tested bacterium.

anti-*M. tuberculosis* activity *in vivo*.

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