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Full Length Research Paper

Determination of the minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) for a novel antibiotic

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A novel antibiotic had a wide range of antibacterial spectrum. It was very stable to heat and to acid and alkali. In addition, the effects of storage on the antibiotic had not been observed even after it had been placed in a 37°C thermostat for up to four weeks. The MICs for the antibiotic against eleven strains of bacteria had been determined. Also, the determinations of the minimum bactericidal concentrations (MBCs) against four bacteria were conducted. The antibiotic-producer (*Bacillus subtilis*) was no better in tolerating its own antibiotic than all the non BS-producers tested.

Key words: Antibiotic, stability, minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs).

INTRODUCTION

Historically, natural products have played a key role in the discovery and development of many antibiotics (Bush, 2004; Shlaes et al., 2004; Newman and Cragg, 2007). Currently, there is a growing recognition of the pressing need for new antibiotic. 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 and Pelzer, 2007; Zakeri and Wright, 2008; Luzhetskyy et al., 2007). At least three of every four current antibacterial agents are related in some way to natural products (Demain, 2009). In the course of screening the bacteria that can antagonize *Xanthomonas oryzae pv.oryzae (*a pathogen of rice), a bacterium had been isolated from the egg plant leaf in the suburb of Hangzhou City, zhejiang Province, People Republic of China. The bacterium could produce an antibiotic which we named as BS. The main purpose of this study was to evaluate BS's agonistic efficiency against other bacteria.

MATERIAL AND METHODS

The culture media and the bacterial strains

KMB culture medium: BBI company, peptone 20 g, glycerol 15 ml, K_2HPO_4 1.5 g, $MgSO_4$, 0.75 g, volume was adjusted to 1000 ml by distilled water, sterilized at 121°C for 20 min; LB culture medium: BD Difco Tryptone 10 g, USB yeast extract 10 g, sodium chloride 5 g, adjusted to pH 7 by 0.1 N NaOH, volume was adjusted to 1000 ml by distilled water, sterilized at 121°C for 20 min;

Quartz sand: Quartz sand was immersed in acidic potassium dichromate solution for 24 h in order for the organic substances to be oxidized that had been captured by the quartz sand (the acidic potassium dichromate solution: K2Cr2O7 37 g plus 300 ml water was heated and stirred until potassium dichromate was completely dissolved. After it cooled down, 300 ml of 98% sulphuric acid was gradually added). The quartz sand was eluted with distilled water for 10 h with the purpose of removing both the metallic ions and the substances that had been oxidized by the acidic potassium dichromate solution. The quartz sand was then sterilized by dry heat at 180°C for 2 h, using an electrothermal constant temperature dry box;

The bacterial strains: BS-producer 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 *Escherichia coli* were kindly provided by The College of Agriculture and Biotechnology of Zhejiang University, P. R. China. All the other bacteria, including *Staphylococcus epidermidis, Streptococcus viridians*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* 25923, *Bacillus subtilis* (non BS-producer), *Shigella dysenteriae*, *Klebsiella pneumoniae*, *Salmonella typhi*, were kindly provided by The College of Life Science, Zhejiang Chinese Medicine University, P. R. China.

Fermentation and isolation of BS

The quartz sand was immersed in KMB culture medium, with the surface of the quartz sand not being covered with liquid culture

Table 1. The stability of BS to heat, acid and alkali and the effects of storage on BS as expressed by the value of the diameter of the inhibition zone.

Heat	Temperature (°C)/ 1 h)	50	80	100	121		
	inhibition zone (cm)	1.3	1.3	1.1	1.0		
	Time (min)/100(°C)	15	30	60	120		
	inhibition zone (cm)	1.3	1.3	1.1	1.0		
Acid and alkaline	pH (37°C, 12 h)	1	5	7	10	12	14
	inhibition zone (cm)	1.2	1.3	1.3	1.3	1.1	1.0
Storage	Time（days) 37(°C）	7	14	21	28		
	inhibition zone (cm)	1.3	1.3	1.3	1.3		
Control		1.3					

medium. BS-producer was cultured in the quartz sand at 37°C for ten days (previous work had shown that the bacterium produced more BS when it was cultured on solid medium than in liquid medium). Thereafter, BS was distilled from the quartz sand that had absorbed BS secreted by the bacteria. The distilled BS was chromatographed on active carbon chromatographic column in order for the active carbon to absorb (remove) the highly lipophilic substances with no bio-activity. Then the active carbon chromatographic column was eluted with ethyl ether. The eluted ethyl ether that contained BS was left at room temperature (25 to 30°C) overnight for the ethyl ether to be evaporated. The remainder was chromatographed on silica gel column, which was eluted with ethyl ether. The fraction with the greatest activity was further chromatographed on silica gel column and then eluted with normal pentane:ethyl ether = 1:4. Both the normal pentane and the ethyl ether were removed by evaporation at room temperature (25 to 30°C). Bio-activity was tracked by anti-*E. coli* activity (agar diffusion method).

The determinations of the stability to heat, acid and alkali and the determination of the effects of storage

Different treatments for BS were as follows: (1) The heat treatment: Aqueous solutions of BS (10 µg/ml) were autoclaved for 1 h at different temperatures (at 50, 80, 100 and 121°C, respectively). In addition, other aqueous solutions of BS (10 µg/ml) were autoclaved at 100°C for different periods (for 15, 30n, 60 and 120 min, respectively); (2) The acid and alkaline treatment: Aqueous solutions of BS (10 µg/ml) were respectively adjusted to pH 1, 5, 7, 10, 12 and 14 with 0.5 N HCl and 0.5 N NaOH. Then, the solutions were left at 37°C for 12 h. After the acid and alkaline treatment having been complete, pH values of all the samples were adjusted to pH 7; (3) The storage treatment: aqueous solutions of BS (10 µg/ml) were stored in a 37°C incubator for different period of time (for one, two, three and four weeks, respectively). After all the above treatments had been respectively conducted, all the samples were respectively put in a -20°C refrigerator for later use. A fresh untreated solution of BS (10 µg/ml) was used as a control. The determinations of the stability of BS to heat and pH, as well as the determination of effects of storage on BS, were conducted by disk diffusion method as described by M. K. Lalitha with minimum modification (M. K. Lalitha, 2004), using *E. coli* as the test organism. Inhibition zones were measured in millimeters. Average of 16 replicates (4 plates × 4 discs) were measured and calculated as shown in Table 1.

The determinations of the MICs (the minimum inhibitory concentrations) and the MBCs (the minimum bactericidal concentrations)

The MICs for BS against all the eleven bacteria were determined using the NCCLS two-fold serial dilution broth method with minimum modification (National Committee for Clinical Laboratory Standards, 2000). The determinations of the MICs were carried out in triplicate and the mean values were used. The 96-well plates were scanned with ELISA reader at 540 nm. The MICs were taken as the lowest concentration of BS that caused optical density reduction by more than 90% compared with control growth results. All the MIC wells, which did not show any turbidity, were streaked on LB agar plates. The lowest concentrations of BS that did not permit any visible growth on the plates after 48 h of incubation at 37°C were recorded as the MBCs.

RESULTS

The stability of BS to heat, acid and alkali and the effect of storage on BS

After the heat treatment, the acid and alkaline treatment and after the storage, the inhibition zones for BS remained almost the same (Table 1). This indicated that BS was very stable.

MICs and MBCs

All the eleven bacteria tested, whether they were gram-positive or gram-negative, were sensitive to BS. The values of the MICs were given in Table 2. It was interesting that the MICs against BS-producer were so close to the MICs against all the non BS-producers tested.

The MBCs for BS against the different bacteria were shown in Table 3. Although, the both *B. subtilis* did not show any turbidity after incubation in LB media that respectively contained 1 × MIC, 20 × MIC, 40 × MIC, 80 × MIC of BS, when they were streaked on LB agar plates,

Table 2. MICs (the minimal inhibition concentration) for BS against bacteria tested.

Bacteria strain	MIC (μg/ml)
Gram-positive	
S. epidermidis	0.1
S. viridans	0.1
S. aureas 25923	0.1
The BS producer (*B.subtilis*)	0.05
The non-BS producer (*B.subtilis*)	0.05
Gram-negative	
P. aeruginosa	0.05
S. dysenteriae	0.1
K. pneumoniae	0.2
S. typhi	0.1
E. coli 25922	0.1
S. dysenteriae	0.1

Table 3. The MBCs for BS against *E. coli* and *S .aureus* (the MBCs against the both *B. subtilis* could not be obtained due to sporulation).

	BS-producer (*B. subtilis*)	**Non BS-producer (*B. ubtilis*)**	***E. coli***	***S. aureu***
BS free	Sporulation rate ≈ 0% Survived	Sporulation rate ≈ 0% Survived	Survived	Survived
1×MIC	Sporulation rate ≈ 100% Survived	Sporulation rate ≈ 100% Survived	Survived	Survived
20×MIC	Sporulation rate = 100% Survived	Sporulation rate = 100% Survived	Killed	Killed
40 × MIC	Sporulation rate = 100% Survived	Sporulation rate = 100% Survived	Killed	Killed
80 × MIC	Sporulation rate = 100% Survived	Sporulation rate = 100% Survived	Killed	Killed

Survived: After being treated by BS, the bacterium could be re-cultured. Killed: After being treated by BS, the bacterium could not be re-cultured.

colony growth appeared on all the plates after incubation. Thus, the MBCs against the both *B. subtilis* had not been obtained. Wirtz-Conklin spore staining confirmed that spores had been formed within the cells of the both *B. subtilis*. Sporulation could be used to explain why the high concentrations of BS had not caused lethal effects on the both *B. subtilis* despite of the fact that the both *B. subtilis* were so sensitive to BS (MIC = 00.5 μg/ml).

DISCUSSION

1. The epidemic of antibiotic-resistant bacteria has spurred renewed interest in finding novel antibiotics (Singh and Barrett, 2006; Butler and Buss, 2005). With a wide range of antibacterial spectrum, BS could be a promising candidate for a new antibiotic.

2. Antibiotic-producing bacteria always employed certain mechanism/mechanisms to avoid suicide by its own antibiotic (Demain, 1974; Cundliffe, 1989; Tatsuo and Makoto, 2003; Hopwood, 2007; Cundliffe, 2010). However, the MICs for BS against all the bacterium tested, including BS-producer, was so close that the answer to whether BS-producer had its special mechanism/mechanisms to deal with its own antibiotic rather than sporulation seemed to be negative. To our

best knowledge, no similar case could be traced in the existing literatures that were about antibiotic-producing bacteria.

3. Experiments had confirmed that even if fermentation time was extended to four months, there was still very limited amount of BS that could be extracted from 2000 ml BS-producer culture (data not shown). Because BS was very stable, the possibility could be ruled out that BS's low productivity was mainly caused by BS's decomposition that might occurred during the period of fermentation. It was well known that end-product inhibition in the fermentation process is a main factor for causing a very level of secondary metabolite, including antibiotic (Haifeng and Kozo, 2001; Haifeng and Kozo, 2001; Cen and Cai, 2000). It stood to reason that it was BS producer's high susceptibility to its own antibiotic that gave rise to a serious end-product inhibition and it was such a serious product inhibition that given rise to a very low level of BS productivity.

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