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Anti-Methicillin Resistant *Staphylococcus aureus* Activity of *Brevibacillus laterosporus* Strain SA14

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ABSTRACT

We isolated strain SA14 that produced an antibacterial agent against *Staphylococcus aureus* and clinical isolates of methicillin-resistant *S. aureus* from air samples and identified it to be *Brevibacillus laterosporus* using API 50 CHB strips. It showed a broad range of antibacterial activity against bacteria in contaminated drinking water such as *Escherichia coli* and *Pseudomonas* and even the opportunistic microorganism *Candida albicans*, when investigated by the cross streak method. It excreted antimicrobial peptides into culture broth on the first day of cultivation. The peptide molecular weight determined by SDS-PAGE was 116 kDa. Characteristic measurements indicate that the peptides had a relatively gram-positive bacteria inhibitory spectrum, especially, *S. aureus* and MRSA, when investigated by agar well diffusion. The anti-MRSA activity was not affected by a wide pH, chemical compounds and temperature range.

Keywords: *Brevibacillus laterosporus*, antibacterial activity, methicillin-resistant *Staphylococcus aureus*

INTRODUCTION

The widespread use of antibiotics has given rise to many mutant resistant strains [1]. Hence, there is a strong need for finding new antibiotics that can combat some new targets in the resistant strains and also could be very selective. Bioactive compounds, produced by probiotic bacteria, have to be developed in order to treat these bacterial infections [2,3], it is one of many different strategies for finding new antimicrobial agents, the area of antibacterial peptides [4]. Currently, there is no universal class of probiotic bacterium although the most common types available are lactic acid bacteria (e.g., *Lactobacillus* spp.). These bacteria are found normally in the gastrointestinal tract (GIT) of humans and animals. A second class comprises those that are not normally found in the GIT, within this group of allochthonous probiotic microbes are the spore-forming bacteria, normally members of the genus *Bacillus*. *Bacillus* probiotic products fall into 2 major groups, those for prophylactic use and those sold as health food supplements or novel foods. *Bona fide Bacillus* species being used include: *Bacillus subtilis*, *B. cereus*, *B. licheniformis*, *B. pumilus*, *B. clausii* and *B. coagulans*. Other spore-formers being used are *Paenibacillus polymyxa* and *Brevibacillus laterosporus*, both being former *Bacillus* species and now belonging to the *Bacillus sensu lato* group. Interestingly, bacteriocins produced by *Bacillus* and other microorganisms are the most promising antibacterial peptides [5,6]. *Brev. laterosporus,* previously classified as *Bacillus laterosporus* [7], is an aerobic spore-forming bacterium characterized by its ability to produce canoe-shaped lamellar parasporal inclusion adjacent to the spore. The species has long been known to include strains toxic to certain invertebrate organisms [8-11]. In addition, some strains of *Brev. laterosporus* produce the medically important substances spergualin [3,12], and bacithrocins A, B and C [13], including, a peptide antibiotic with cyanolytic activity [14]. In this study, we report a strain of *Brev. laterosporus*, SA14, an environmental isolate that, interestingly, produced some antibacterial agents that inhibit the growth of a number of pathogenic bacteria. The characterization of the corresponding anti-methicillin resistant *S. aureus* agent, culture broth from SA14, was elucidated.

MATERIALS AND METHODS

Media and chemicals

Luria-Bertani (LB: HIMEDIA) agar media was used in the study as an antibiotic-producing medium. Mueller-Hinton (M-H: Merck) was used for plating of the clinical strains of methicillin-resistant *S. aureus* (MRSA) and also to carry out the anti-MRSA assay.

Growth conditions for antimicrobial production

The anti-MRSA agent producing SA14 was grown in LB medium. Initially, the seed inoculum was prepared in a shake tube by transferring only 1 colony from an LB

plate in 5 ml of LB medium for 24 h at 37 °C and 150 rpm. The 2 % seed culture, correlated to standard McFarland No.0.5, was transferred to a 250 ml flask with 40 ml of LB medium. The experiment was carried out in duplicate. The culture flask was incubated at 37 °C for 5 days at 150 rpm. After every 24 h, 1 ml of the culture was drawn from each flask and centrifuged at 10,000 *g* to pellet the cells, and the concentration of the protein in culture broth was assayed by Bradford as described elsewhere [15], using a Test Kit (Bio-Rad). The regular sample (culture broth) was collected after 24 h and was used for the anti-MRSA assay.

Isolation and identification of strain SA14

SA14 was isolated from an air sample at Walailak University, by using an air sampler (microflow 90). The isolate SA14 was examined for production of acid from 49 compounds as a sole carbon source using a API 50 CHB Test Kit (Bio-merieux) according to the manufacturer's instructions. The results were analyzed with API Plus software (API 50 CHB V 3.0: Bio-merieux).

Test microorganisms

The clinical isolates of MRSA were collected from patients with skin and soft tissue infections in the Maharaj Nakhon Si Thammarat Hospital, Thailand. In addition to MRSA, 4 other indicator strains obtained from Thailand Institute of Scientific and Technological Research (TISTR), i.e., *S. aureus* TISTR 517, *Escherichia coli* TISTR 887, *Pseudomonas aeruginosa* TISTR 781 and *Candida albicans* TISTR 5779, were used for evaluating the sensitivity against SA14. These isolates were identified by using conventional laboratory methods [16,17].

Antimicrobial activity assay

In vitro antibacterial activity of SA14 was tested against indicator strains by cross streak technique as described in Lemos *et al* [18].

Only anti-MRSA activity was measured for 5 days by agar well diffusion assay with plates overseeded with representative strains of MRSA as described by Cintas *et al* [2]. Agar wells were filled with 80 µl serial 2-fold dilutions of culture broth and corresponding plates were incubated at 37 °C overnight. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution yielding a definite zone of inhibition on the MRSA lawn.

Effect of enzymes, heat, pH, and surfactants

The culture broth medium (274 µg/ml protein) was incubated at 37 °C with proteinase K, pronase, trypsin, chymotrypsin, lipase, lysozyme or amylase (Sigma) to a final concentration of 1 µg/ml for 3 h at room temperature, or incubated at −20 °C for 1 day. The residual anti-MRSA activity was determined by dilution as described above. To determine the effect of heat at different pH values, the culture broth medium (274 µg/ml protein) was adjusted to pH 2.0, 7.0 or 10.0 using 1 M HCl or 1 M NaOH. These

were then incubated either at 37 °C for 5 h or at 121 °C for 15 min, readjusted to pH 7.0 for assaying the residual anti-MRSA activity. The effect of SDS, Tween 80, Triton X-100, all at 1 % (v/v), EDTA (10 mM) was determined by incubating the culture broth with any of these reagents at 37 °C for 5 h. The sample then was diluted 10-fold and assayed for residual anti-MRSA activity. Untreated culture broth was used as control.

Molecular weight determination

The culture broth was loaded on Vivaspin 20 (Vivascience, Sartorius) cut-off at 3 kDa, and centrifuged at a specific rpm and time as per the instructions given in the manual. The concentrated-culture broth was checked for anti-MRSA activity by swab-paper disc diffusion. Approximately 1×10^6 cells of the MRSA strains grown in M-H medium were used for testing the anti-MRSA activity [19].

SDS-PAGE

Most of the studied antibacterial agents are found to be proteins or conjugates of proteins. In order to study the nature of the anti-MRSA agent that was secreted in the media by the SA14 strain, the concentrated-culture broth was collected from the first day as described above, and the protein profile was studied through SDS-PAGE [20]. A 12 % resolving gel and a 4 % stacking gel were used along with the prestained SDS-PAGE broad range molecular weight standard (Bio-Rad).

RESULTS

Strain identification

SA14 producing anti-MRSA agents isolated from air samples were used in this study. Biochemical tests using API 50 CHB showed that SA14 could utilize glycerol, esculin; produce acid from D-ribose, D-glucose, D-mannose, D-mannitol, N-acetylglucosamine, salicine, D-maltose, D-trehalose. The identification result showed 99.9 % identity to *Brev. Laterosporus*, SA14 was then identified as *Brev. laterosporus*.

Antibacterial spectrum

The spectrum activity of SA14 was tested by cross streaking against 4 indicator strains as mentioned above, including 25 clinical isolates of MRSA. The variation in the degree of inhibition activity was observed in the tested strains. It was very clearly seen with respect to the decreasing growth in the case of *S. aureus* TISTR 517 and MRSA strains.

Anti-MRSA assay

The anti-MRSA production by SA14 was found after 24 h of incubation and remained up to the fifth day of the preliminary experiment. After every 24 h, the culture broth from the SA14 culture flask was tested for its anti-MRSA activity. An initial zone of inhibition was observed on the first day, and remained constant for anti-MRSA

activity for at least 5 days with a zone of inhibition measuring in range of 16 - 18 mm for 80 μl of culture broth.

Sensitivity to heat, pH, enzymes, and surfactants

The anti-MRSA activity of the culture broth was 50 % lower than the anti-MRSA activity for all the heat treated samples (121 °C). In context with the different pH values (2.0, 7.0 or 10.0) that were used, the anti-MRSA activity remained unaffected at both pH (2.0 or 7.0), but was decreased 50 % at pH 10.0. The anti-MRSA agent was sensitive to digestion with different enzymes, especially after incubating at −20 °C for 1 day. The anti-MRSA agent retained its full activity following exposure to SDS, Tween 80, Triton X-100 and EDTA (**Table 1**).

Table 1 Effect of different enzymes, heat, pH and surfactants on inhibitory activities of culture broth from strain SA14.

Treatment	**Inhibitory activities of culture broth (AU/ml)**
Enzymes	
Control	200
Amylase	100
Lipase	100
Lysozyme	100
Chymotrypsin	100
Pronase	100
Proteinase K	100
Trypsin	200
pH and Temperature (°C)	
Control	200
2.0 and 37	200
2.0 and 121	100
7.0 and 37	200
7.0 and 121	100
10.0 and 37	100
10.0 and 121	100
Surfactants	
Control	125
SDS	125
Tween 80	125
Triton X-100	125
EDTA	125

Molecular weight determination

The culture broth obtained using the 3 kDa cut-off concentrator tube (Vivaspin 20) was tested for anti-MRSA activity. The activity was clearly seen in case of this cut-off tube, suggesting that the molecules having higher molecular weight than the cut-off limit were retained in the tube, indicating that the anti-MRSA molecule must have a molecular weight of more than 3 kDa.

SDS-PAGE

The proteins that were secreted in the LB media by SA14 were shown by SDS-PAGE. The anti-MRSA activity was observed after 24 h and concentrated by using a concentrator tube. Only a single band with a molecular weight of 116 kDa was seen for this culture broth (**Figure 1**).

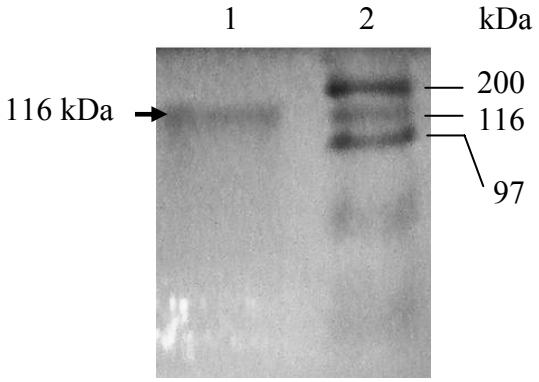

Figure 1 SDS-PAGE showing the protein profile of antibacterial culture broth of *Brevibacillus laterosporus* strain SA14. Lane 1 shows the single protein band for the antibacterial culture broth obtained on the first day using the 3 kDa cut-off concentrator tube (Vivaspin 20) and lane 2 shows the broad range molecular standard.

DISCUSSION

In this study, the extracellular antibacterial agent was shown to accumulate in the growth medium as seen on the first day of the culture period. The activity of this antibacterial agent was most effectively seen against gram-positive bacteria, *S. aureus* and MRSA strains. Also, indicator strains were chosen to represent potential common water-borne pathogens, such as *E. coli* [21,22] as well as strains important for opportunistic infections. These were also sensitive to SA14 tested by the cross streaking. An attempt was made to concentrate this antibacterial agent using a Vivaspin 20 concentrator tube, able to concentrate 3.5-fold of the antibacterial agent present in a 1 day old culture broth. Its inhibitory activity showed similar patterns of spectra as non-concentrated culture broth. The conventionally used ammonium sulfate precipitation

method was able to precipitate the antibacterial agent present in the culture broth (data not shown).

The inhibitory activity produced by *Brev. laterosporus* SA14 was decreased upon treatment with proteolytic enzymes such as, chymotrypsin, pronase or proteinase K, but was not affected by trypsin. Surprisingly, non-proteolytic enzymes reduced the inhibitory activity. Some strains, especially in the *Bacillus* genus, often produce a series of nonribosomal peptide isoforms, contained unusual residues, such as amino acids of formylated, acylated, and covalent linked to another function group in nonribosomal peptide gramicidin, surfactin, iturin and tauramamide [16,23-26], as well as post translational modified amino acid in ribosomal peptide subtilin and sublactin [27,28]. Therefore, the loss of inhibitory activity after exposure to enzymes, might be the result of the presence of one or more of these elements, they could also interfere with peptide sequencing [29]. The antibacterial activity is the most stable at pH 7.0 after incubating at 37 °C for 5 h, but its inhibitory activity was still observed at pH 2.0 or 10.0 after incubating at 37 °C or 121 °C. The lower pH give rise to the higher stability of antimicrobial agent to high temperature, similar to previous findings for nisin [16,30,31].

In conclusion, the strain SA14 appears to have a potential to produce an anti-MRSA agent, which is resistant to acidic or basic environments. Stability of the anti-MRSA agent to heat is considered to be very important. These characteristics make it a good candidate for the food industry and human health in the future.

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บทคัดย่อ

อภิญญา ชูพันธุ์ กฤษณวัฒ นาคบุตร กุ้ง ดาววีระกุล กิจติศักดิ์ ชววิสิฐ และ มณฑล เลิศคณาวนิชกุล

ฤทธิ์ต้านการเจริญสแตปฟิลโลคอคคัสออเรียสที่ดื้อยาเมธิซิลลินของเชื้อบรีวิบาซิลลัสเลเตอโรสปอรัสสายพันธุ์ SA14

คณะผู้วิจัยสามารถแยกเชื้อสายพันธุ์ SA14 ได้จากอากาศโดยพบว่าเชื้อสามารถผลิตสารต้านการเจริญสแตปฟิลโลคอคคัสออเรียสและสแตปฟิลโลคอคคัสออเรียสที่ดื้อยาเมธิซิลลิน ซึ่งสามารถวินิจฉัยแยกชนิดเชื้อดังกล่าวด้วยการดูความสามารถในการใช้คาร์โบไฮเดรตต่างๆ 49 ชนิดของชุดทดสอบ API 50 CHB และวินิจฉัยได้เป็นบรีวิบาซิลลัสเลเตอโรสปอรัส สายพันธุ์ SA14 นอกจากนี้เมื่อนำไปทดสอบด้วยวิธีขีดเชื้อพบว่าเชื้อสามารถต้านการเจริญของเชื้อที่มักพบปนเปื้อนในแหล่งน้ำ ได้แก่ เอชเชอริเชียคอไล ซูโดโมแนส รวมไปถึงเชื้อก่อโรคฉวยโอกาสได้แก่ แคนดิดาอัลบิแคน และพบว่าเชื้อดังกล่าวสามารถปล่อยสารที่มีฤทธิ์ต้านการเจริญของเชื้อดังกล่าวออกไปยังน้ำเลี้ยงเชื้อได้ตั้งแต่วันแรกของการบ่มเพาะเลี้ยงเชื้อ โดยพบว่าเป็นสายเปปไตด์ที่มีขนาดโมเลกุล 116 กิโลดาลตัน เมื่อทดสอบด้วยวิธีโซเดียมโดดิซิลพอลิอะคริลาไมด์เจลอิเล็กโตรฟอริซิส และยังพบว่าเปปไตด์ดังกล่าวมีฤทธิ์ในการต้านการเจริญของแบคทีเรียแกรมบวกได้เป็นอย่างดี โดยเฉพาะสแตปฟิลโลคอคคัสออเรียสและสแตปฟิลโล-คอคคัสออเรียสที่ดื้อยาเมธิซิลลินเมื่อทดสอบด้วยวิธีซึมผ่านเนื้อวุ้น โดยฤทธิ์ดังกล่าวมีความทนทานต่อความเป็นกรด-ด่าง สารเคมี และช่วงอุณหภูมิที่ใช้ในการทดสอบ กล่าวคือเปปไตด์ดังกล่าวยังคงแสดงฤทธิ์ต้านการเจริญของเชื้อสแตป-ฟิลโลคอคคัสออเรียสที่ดื้อยาเมธิซิลลินได้เช่นเดิมภายหลังจากนำไปผ่านปัจจัยทดสอบดังกล่าวข้างต้น

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