Antagonistic Activity of *Bacillus* Bacteria against Food-Borne Pathogens

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Abstract

*Bacillus* bacteria have attracted the attention of scientists as promising probiotics because of their versatile antimicrobial activity and established health benefits on the host. In this study, seven *Bacillus* strains were identified and analyzed for antagonistic activity against broad spectrum of food borne pathogens. All strains were identified as *B. subtilis*, based on the results of morphological, biochemical characterization and 16S rDNA sequence analysis. *B. subtilis* strains demonstrated antagonistic activity against test-cultures of pathogens, including multiresistant strains. Reference *Bacillus* strains, derived from the commercial probiotics did not show antagonistic activity against tested strains of pathogens. Three the most active cultures were studied for production of biosurfactants. Crude biosurfactants were isolated and analyzed by oil spread test and inhibition activity against *Salmonella*, *Shigella* and *Staphylococcus* cultures. Biosurfactants from three tested *B. subtilis* strains gave positive oil spread test. Inhibition activity of biosurfactants was found only against *Staphylococcus* strains. Production of biosurfactants depended on the incubation conditions of *Bacillus* culture. Best results were obtained after cultivation of bacilli in starch broth at 30°C. The concentration of produced biosurfactant increased in time with growth of bacteria and reached the maximum at 30 hours of incubation.

Keywords: *Bacillus subtilis*; Antagonistic activity; Food borne pathogens; Biosurfactants

Abbreviations: MRSA: Methicillin Resistant *Staphylococcus aureus*; SA - Starch Agar; TBS - Trypticase Soy Agar; NA - Nutrient Agar; CFU – Colony Forming Unit; OD\(_{600}\) – Optical Density at a wavelength of 600 nm

Introduction

Foodborne pathogens are among the most significant problems in maintaining the health of the population. In 2011 the CDC estimates that each year roughly 1 in 6 Americans (or 48 million people) gets sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases. The leading causes of foodborne illnesses in the United States are *Salmonella* and *Shigella* [1,2]. *Staphylococcus aureus* is among top five pathogens contributing to domestically acquired foodborne illnesses. Staphylococcal food poisoning is estimated to account for 241,148 foodborne illnesses per year in the United States, according to the CDC information (http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html). Foodborne illnesses are routinely treated with several classes of antibiotics. However, the use of these antibiotics has become problematic as over the years there have been numerous reports of cases of multi-antibiotic resistant food borne pathogens, worldwide [3-5]. In the United States, the proportion of methicillin-resistant *S. aureus* (MRSA) isolates from patients in intensive care units increased from 1992 to 2003 by 3% per year. Moreover, there is a great concern that the continued use of these drugs will result in the emergence of new resistant strains of these bacteria [6-9]. Colonization of the intestinal tract with MRSA may have important clinical implications, such as development of antibiotic-associated diarrhea, environmental dissemination, subsequent risk of infections and toxic shock syndrome [10-14]. Since foodborne infections have a dramatic impact on morbidity and mortality, particularly of infants and children, new approaches for cost effective and easy-to-deliver prophylaxis and treatment of these infections are highly desirable.

One of the growth areas in the control of foodborne infections is the use of probiotics [7]. Probiotic prophylaxes and therapies are gaining wider acceptance as more scientific data emerge regarding the interaction between pathogen and beneficial microbes in the human intestinal tract and molecular mechanisms of probiotics’ action. Probiotic bacteria which confer beneficial effect for the host and have pronounced antagonistic activity against these pathogens are expected to present a clear alternative in the prevention and treatment of foodborne infections.

Bacteria of the *Bacillus* genus possess a great potential as probiotic cultures. *Bacillus* bacteria are among the most widespread microorganisms in nature. These bacteria are known to be producers of more than 200 antibiotics. *Bacillus* antibiotics differ in their structure, as well as spectrum of activity [15]. Some strains of *Bacillus* synthesize bacteriocines, which are only effective against bacteria of the same species, others produce antibiotics against Gram-negative bacteria and still other strains have a wide spectrum of antibiotic activity (including antifungal and antiprotozoan) [16]. Thus, it is possible to find strains with unique spectrum of activity among *Bacillus* bacteria. The aim of this work was to isolate and characterize *Bacillus* strains with pronounce activity against food borne pathogens.

Materials and Methods

Bacterial strains

Seven bacterial strains (16k, M1-1, 11-89, M2-3, 101, BSB, 105), isolated from environment, were used in this study. Morphological characterization of the cultures was done with high resolution CitoViva microscope [17,18]. Gram reaction and catalase activity were analyzed...
in tested strains. Bacterial cultures were identified using API 50 CHB tests (bioMerieux, Marcy-l’Etoile, France). For further characterization, the 16S rRNA gene was PCR amplified using universal 16S primers that correspond to positions 900SF and 5031R. Products of sequencing reactions were analyzed with an ABI 3100+ AVANT Genetic Analyzer in MIDI Labs (Newark, DE). Sequence analysis was performed using BLAST and Sherlock® DNA microbial analysis software and database.

**Probiotic strains**

_Bacillus_ cultures from commercial probiotics were studied as the reference strains (Table 1).

**Test-cultures**

Test-cultures of _Salmonella_, _Shigella_ and _Staphylococcus_ were obtained from the collection culture of Auburn University (Auburn, AL). Stock cultures were maintained at -20°C in NZY medium, supplemented with 25% (v/v) glycerol.

**Antagonistic activity of Bacillus strains**

Activity of _Bacillus_ strains against pathogens were studied by the method of delayed antagonism in solid nutrient medium [19]. Briefly, _Bacillus_ strains were inoculated as a line on the surface of a nutrient media. After 72 h of growth at 30 or 37°C overnight test-cultures were inoculated as a perpendicular line to the _Bacillus_ culture. The plates were incubated for 24 h at 37°C. The antagonistic activity was detected as a zone of pathogens’ growth inhibition. Different media were tested to assess the antagonistic activity of _Bacillus_ strains–NZY, starch agar (SA), trypticase soy agar (TBS) and nutrient agar (NA). Starch agar composed of starch (10 g/L), peptone (5 g/L), NaCl (0.5 g/L), agar (15 g/L) was used previously for cultivation of _Bacillus_ probiotic strain [20]. Test-cultures of pathogens were grown overnight in NZY medium at 37°C.

**Biosurfactant evaluation**

For preparation of the inoculum, _Bacillus_ strains were grown in NZY medium overnight at 37°C on shaker-incubator (200 rpm). Seed cultures were inoculated into nutrition media (1 mL of overnight culture into 250-mL flack with 50 mL of tested medium). Two media were used for cultures cultivation: SA and a fermentation media for biosurfactant production by _B. subtilis_ natto [21]. _B. subtilis_ strain was cultivated in 250 mL flasks with 50 mL of starch medium at 30°C for 32 hours. At different time intervals, the fermentation medium was sampled for determination of biomass and biosurfactant concentration.

Surface activity of biosurfactants was measured by an oil spreading test [22,23]. Briefly, 20 µL of crude oil was added to a Petri dish (90 mm diameter) with 50 mL of distilled water to form a thin membrane. Ten microliters of sample was put onto the center of the oil membrane. The diameter of the oil-displaced circle area was measured. Each sample was tested in triplicate.

Antimicrobial activity of biosurfactants was evaluated by an agar well diffusion method [20]. Prepared suspensions of test-cultures in PBS (10° CFU/mL) were inoculated onto the surface of agar medium (100 µL of suspension on each plate). Wells (6 mm diameter) were made with a sterile cork borer. 50 µL of the test solutions were added to each well. Plates were incubated for 24 hours at 37°C. Zones of test-cultures growth inhibition were measured.

**Statistics**

Statistical analyses (t- Test and ANOVA) were performed using Microcal™ Origin® version 6.0 (Northampton, MA) to validate the signification of the results. The data are presented as means (± SD) of at least three replicates.

**Results**

**Identification of Bacillus strains**

The microscopic study of bacterial cultures showed these strains to be Gram-positive rods, less than 1 µm in diameter. All strains sporulated aerobically without swelling of the cell and produced catalase. These data indicated that tested strains belong to _Bacillus_ genus. Additional testing with API 50CHB kit resulted in identification of all cultures as _B. subtilis_. Partial sequence of 16S rRNA gene confirmed the obtained results of biochemical identification.

**Antagonistic activity**

Antagonistic activity of _B. subtilis_ cultures was tested on different nutrient media at two temperatures: 30° and 37°C. All cultures showed prominent growth on selected media at both temperature, but no antagonistic activity was indicated at 37°C. _Bacillus_ cultures inhibited the growth of pathogenic bacteria only after growth on SA at 30°C (Table 2).

Strains BSB, 16K and 105, showed the highest antagonistic activities, were used in further experiments with broad spectrum of _Salmonella_ and _Staphylococcus_ strains, including clinical isolates. _Bacillus_ cultures from commercial probiotics were tested as reference strains. Antagonistic activity of bacilli was studied after growth on SA at 30°C. _B. subtilis_ strains were highly effective against all tested strains of _Salmonella_ and _Staphylococcus_ (Table 3, Figures 1 and 2). Commercial _Bacillus_ strains showed no antagonistic activity against test-cultures.

**Biosurfactant production**

Biosurfactant production was tested in two media–starch broth and fermentation medium, used for surfactant production by _B. subtilis_ natto [21]. _B. subtilis_ strains were incubated in two medium at 30°C and 37°C. Production of biosurfactant was assessed by the oil spreading technique and by inhibition of test-cultures growth.

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**Table 1: Characterization of Bacillus probiotic strains.**

<table>
<thead>
<tr>
<th>Bacillus strain</th>
<th>Probiotic</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cereus</em> IP 5832</td>
<td>Bactisubtil</td>
<td>Cassenne Marion, Paris, France</td>
</tr>
<tr>
<td><em>B. cereus</em> DM-423</td>
<td>Cereobiogen</td>
<td>Keda Drugs Trade Co Ltd under Dalian university of Medical Sciences, China</td>
</tr>
<tr>
<td><em>B. clausii</em></td>
<td>Enterogermina</td>
<td>Sanofi -Synthelabo, Milan, Italy</td>
</tr>
</tbody>
</table>

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anti-Salmonella activity of Bacillus strains.

<table>
<thead>
<tr>
<th>#</th>
<th>Test -cultures</th>
<th>Zone of test-cultures growth inhibition, mm</th>
<th>105</th>
<th>BSB3</th>
<th>16 k</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S. typhimurium Health 9491</td>
<td>0</td>
<td>23.8 ± 0.7</td>
<td>22.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>S. typhimurium DT 104 Dairy</td>
<td>25.6 ± 0.3</td>
<td>25.8 ± 0.8</td>
<td>24.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>S. diarrhea</td>
<td>25.3 ± 0.8</td>
<td>27.1 ± 0.9</td>
<td>26.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>S. panama SA 3583</td>
<td>22.6 ± 0.3</td>
<td>25.3 ± 0.3</td>
<td>23.1 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>S. indica SA 4401</td>
<td>23.1 ± 0.2</td>
<td>25.6 ± 0.7</td>
<td>25.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>S. derby SARB 10</td>
<td>30.2 ± 1.3</td>
<td>31.4 ± 0.8</td>
<td>27.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>S. typhimurium LT2</td>
<td>30.1 ± 0.6</td>
<td>32.3 ± 0.7</td>
<td>28.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>S. mission</td>
<td>26.6 ± 0.3</td>
<td>25.8 ± 0.6</td>
<td>24.3 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>S. montevideo</td>
<td>25.0 ± 0.0</td>
<td>25.3 ± 0.7</td>
<td>23.0 ± 0.6</td>
<td></td>
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<tr>
<td>10</td>
<td>S. typhimurium 6787</td>
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<td>24.3 ± 0.7</td>
<td>19.7 ± 0.8</td>
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<tr>
<td>11</td>
<td>S. typhimurium Heath 1390</td>
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<td>21.7 ± 0.3</td>
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<tr>
<td>12</td>
<td>S. bongori SA 4910</td>
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<td></td>
</tr>
<tr>
<td>13</td>
<td>S. typhimurium Nal 1x fecal</td>
<td>19.7 ± 0.8</td>
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<td></td>
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<tr>
<td>14</td>
<td>S. minnesota</td>
<td>30.7 ± 0.3</td>
<td>31.0 ± 0.7</td>
<td>27.7 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>S. salmonae SA 41106</td>
<td>10.0 ± 0.7</td>
<td>23.6 ± 0.3</td>
<td>18.7 ± 0.9</td>
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<tr>
<td>16</td>
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<td>22.3 ± 0.6</td>
<td>24.0 ± 0.6</td>
<td>21.6 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>S. Thompson 265-4</td>
<td>25.1 ± 0.3</td>
<td>26.3 ± 0.9</td>
<td>23.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>S. infantis SARR 27</td>
<td>31.1 ± 0.9</td>
<td>30.3 ± 0.6</td>
<td>27.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>S. paratyphiurium</td>
<td>30.2 ± 0.3</td>
<td>30.3 ± 0.6</td>
<td>29.6 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>S. typhimurium DT 104 Swine</td>
<td>21.1 ± 0.2</td>
<td>24.6 ± 0.3</td>
<td>22.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>S. typhimurium 9693</td>
<td>22.3 ± 0.3</td>
<td>25.7 ± 0.6</td>
<td>23.3 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3:** Anti-Salmonella activity of Bacillus strains.

best conditions for biosurfactant production for all Bacillus cultures were cultivation in starch broth at 30°C. Results in Figure 3A indicate that oil spread test for biosurfactants, produced by B. subtilis BSB3 and 16k at 30°C gave higher results than for Bacillus cultures, grown at 37°C. Study of inhibition activity of these biosurfactants against test-cultures, showed that activity of biosurfactant from B. subtilis BSB3, cultivated at 30°C was more pronounced, than at 37°C (Table 4; Figure 3B). Biosurfactants from B. subtilis 16k, incubated at different temperatures, demonstrated more consistent results. B. subtilis 105 produced biosurfactant, as it was confirmed by the oil spreading test, but this biosurfactant had lack of inhibition activity. Biosurfactants, produced by Bacillus strains demonstrated inhibition activity only against Staphylococcus cultures. Tested cultures of Salmonella and Shigella were resistant to these biosurfactants.

**Production of biosurfactant during B. subtilis BSB3 cultivation**

Production of biosurfactant by B. subtilis BSB3 increased in
In the present study, newly isolated Bacillus strains were analyzed. All strains, identified as *B. subtilis*, were tested for their activity against Salmonella, Shigella and Staphylococcus strains. Antagonistic activity of Bacillus strains was detected only after cultivation on starch agar at 30°C. Incubation of bacilli on starch agar at 37°C, as well as on NZY, NA and TSA at 37°C and 30°C did not result in antagonistic effect. These outcomes are in accordance with our previous findings about conditions for production of antimicrobial compounds by Bacillus cultures [27]. Three *B. subtilis* strains, showed the highest activity against tested pathogens, were studied with broad spectrum of Salmonella and Staphylococcus cultures, including clinical multi-resistant strains. As reference strains, commercial Bacillus probiotic cultures from Bactisubtil, Cereobiogen and Enterogermina were used. None of the reference strains were active against tested pathogens. *B. subtilis* isolates demonstrated high activity of test-cultures’ inhibition. Antagonistic activity was detected against all strains of Salmonella and Staphylococcus, including MRSA. Inhibition of MRSA by Bacillus cultures was shown by other authors [28-30], but no anti-Salmonella effect was found in the same strains.

Bacteria of the Bacillus genus (predominantly, *B. subtilis*) produce various biosurfactants, which have a high potential for biotechnology and pharmacology [31]. These compounds vary in structure and spectrum of activity and usually are responsible for antimicrobial effects of *Bacillus* bacteria [21,32]. In our study *B. subtilis* strains produced biosurfactants after cultivation in starch broth at 30°C. Incubation of these cultures in fermentation medium, used for *B. subtilis* natto [21], resulted in lack of biosurfactants production. Presence of biosurfactant in cultivation medium was tested by the oil spread test and by inhibition activity against *Salmonella* and *Staphylococcus* strains. It was shown elsewhere, that the oil spread test correlates with the biosurfactant production [23]. Biosurfactants from three tested *B. subtilis* strains gave positive oil spread test, showing the diameter of oil displacement from 3 to 7 mm. These results are in accordance with data for crude biosurfactants from *B. subtilis* natto [21] and from *B. subtilis* and *B. licheniformis* [33]. Inhibition activity of biosurfactants was found only against *Staphylococcus* strains and depended on the incubation temperature of *Bacillus* culture. Biosurfactant from *B. subtilis* BSB3, incubated at 30°C, demonstrated higher activity against

**Discussion**

*Bacillus* bacteria are known to be effective antagonists of different pathogens [15,24]. In recent years bacilli were extensively studied as probiotics, due to their health benefits on the host [25,26]. A search for new Bacillus strains with pronounced antagonistic activity against food borne pathogens opens up promising expectations for treatment of these infections.
Staphylococcus sp. strain B38 newly isolated from soil. Appl Biochem Pharmacology and Department of Biological Sciences of Auburn University for biotechnology and pharmacology.

Further study of antimicrobial compounds, produced by Bacillus bacteria, will result in better understanding of the mechanisms of antagonistic activity of bacilli and selection of new strains, promising for biotechnology and pharmacology.

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References


