# Isolation and Structural Elucidation of a Novel Lipopeptide Brevibacillin from *Brevibacillus laterosporus*

Xu Yang[a]

[a]Department of Food Science and Technology

## Abstract

A soil microorganism was discovered to be active against food spoilage, foodborne pathogen and antibiotic resistant strains, especially Gram-positive bacteria. The producer strain was designated OSY-$I_1$ and identified as *Brevibacillus laterosporus* based on morphological, biochemical and genetic analyses results. The antimicrobial agent produced from OSY-$I_1$, after extraction with isopropanol and resuspended in 50% acetonitrile, was purified using high performance liquid chromatography (HPLC). Fractions from HPLC were collected and tested for their antimicrobial activity. Fractions with highest purity and strongest antimicrobial activity were subsequently analyzed by mass spectrometry (MS) and nuclear magnetic resonance (NMR). As a summary of both the MS and NMR results, the structure of a novel linear lipopeptide, designated brevibacillin, was elucidated. Brevibacillin contains 13 amino acid residues with a $C_6$-fatty acid chain (FA), 2-hydroxy-3-methylpentanoic acid, attached at the N-terminal. Brevibacillin has a molecular weight of 1583.0794 with three modified amino acid residues: α,ß-didehydrobutyric acid, ornithine, and valinol. Brevibacillin remains all antimicrobial activity after treated at 80 ℃ for 60 min and retained at least 50% activity after incubated at pH 3.0 and pH 9.0. Brevibacillin also showed strong antimicrobial activity aginst *Alicyclobacillus* spp., *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus* indicating the potential ability of applying breviacillin to food

industry and clinical field.

**Introduction**

Any change of a food product that renders it unacceptable, from a sensory point of view, is considered a food spoilage event. Food spoilage may be described as any physical damage, chemical change (discoloration, oxidization), or microbial deterioration of product quality. Microbial spoilage by bacteria/fungi is one of the most common food spoilage (Gram et al., 2002). For instance, according to Natural Resources Defense Council, in United Kingdom, around 2/3 of the household food waste is due to food spoilage, especially microbial spoilage (2).

Compared to food spoilage, a much more severe consequence of microbial contamination is foodborne illness. It is, by definition, any diseases caused by consumption of a food product. In many cases, foodborne illness is caused by the consumption of pathogenic bacteria or their toxins. According to Centers of Disease Control (CDC), *Salmonella* spp. and *Clostridium perfringens* cause 11% and 10% of foodborne illness cases in the United States, respectively; and *Salmonella* spp. caused 28% of foodborne illness resulting in death in 2011 (Scallan et al., 2011).

It is obvious that antimicrobial agents are needed to combat against spoilage and foodborne illness pathogens. However, with the emerging occurrence of antibiotic-resistant microorganisms, antimicrobial agents are also urgently needed in the clinical field. In the year of 2013, for the first time, CDC classified drug-resistant bacteria into three categories: urgent threats, such as *Clostridium difficile* and carbapenem-resistant *Enterobacteriaceae*; serious threats including vancomycin-resistant *Enterococcus* (VRE) and methicillin-resistant

*Staphylococcus aureus* (MRSA); and concerning threats, for instance, vancomycin-resistant *Staphylococcus aureus* (CDC, 2013). These antibiotic-resistant bacteria, according to CDC, has affected more than two million people every year and caused death of 23,000 people as a direct result, not including people died from the other situations that were exacerbated by these antibiotic-resistant bacteria (CDC, 2013).

It is very clear that the discovery and application of new antimicrobial agent are of paramount importance. Despite the fact that novel antimicrobial agent may be rare to find, new antimicrobial agents can be discovered from potential producing microorganisms after screening and isolation processes (He *et al.*, 2007; Guo *et al.*, 2012). In this study, we report that the discovery of a linear lipopeptide isolated from a soil microorganism, which are very active against selected indicators (MIC < 4 μg/ml, including food spoilage, foodborne illness and antibiotic resistant strains).

**Materials and Methods**

**Strain screening.** Fermented food samples were collected from local grocery stores. Environmental samples were collected from the campus of The Ohio State University, Columbus, Ohio. Briefly, environmental or food subsamples (10 g each) were homogenized in 0.1% peptone water using a stomacher for 2 min. Serial dilutions were made from the homogenate and aliquot (100 μl) from each dilutions were spread-plated onto trypticase soy agar (TSA; BD Diagnostic Systems, Sparks, MD). After incubation at 37 ℃ for 48 hours, around 1,500 colonies were tested for their capabilities of producing antimicrobial agent. Generally speaking, portions of the colonies were picked by sterile toothpicks onto new TSA plates for growing. After incubation at 37 ℃ for 48 hours, soft TSA agar (0.75%)

pre-inoculated with overnight *Listeria innocua* ATCC 33090 were directly overlaid on top of the testing strains. The overlaid plates were further incubated at 37 ℃ for 16 hours and were inspected for clear zones of inhibition against *L. innocua*. One isolate (designated as OSY-$I_1$) demonstrates a clear zone (diameter greater than 3.0 cm) of inhibition against *L. innocua*. Based on its strong antimicrobial activity, OSY-$I_1$ was selected for further analysis

**Cultures and media.** TSA was used to propagate the new isolate, OSY-$I_1$. For glycerol stock preparation, an overnight culture of OSY-$I_1$ in trypticase soy broth (TSB) was mixed with 80% sterile glycerol in a 1:1 ratio and stored at -80 ℃. Other strains used for antimicrobial spectrum assay are shown in Table 1.

**Strain identification.** The characteristic cellular morphology of OSY-$I_1$ was examined by Gram staining, and spores were stained with malachite green. The hemolytic property of bacterial strain was tested using tryptic soy agar with sheep blood (TSA-SB). The biochemical characteristics of OSY-$I_1$ were examined using a commercial kit (API 50 CH test strips; bioMérieux, Inc., Durham, NC), following kit manufacture protocols. For genetic analysis, 16S rRNA gene sequencing technique was applied (Drancourt *et al.*, 2000). Basically, a commercial kit (DNeasy blood and tissue kit; Qiagen, Valencia, CA) was used to extract the genomic DNA of OSY-$I_1$. Then, universal primers (16S Forward Primer: 5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3' and 16S Reverse Primer: 5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3') were used to target 16S rRNA gene sequence. Finally, by sequencing the PCR product of 16S rRNA and comparing to database (Ribosomal Database Project-II; http://rdp.cme.msu.edu), the species of the producer strain can be identified.

**Extraction and purification of antimicrobial agents from OSY-$I_1$.** Overnight liquid culture of OSY-$I_1$ in TSB was spread-plated on TSA plates. After incubation at 37 ℃ for 72 hours, bacterial cells were scraped into 50 ml centrifuge tubes by using a microscopic slide. Then, isopropanol was added to the centrifuge tube at a ratio of 4:1 (v/w). The centrifuge tubes were then agitated and centrifuged. Supernatant were collected and separated by using a high performance liquid chromatography equipped with an analytical reverse-phase column (5 μm particle size, 250×4.6 mm, Biobasic $C_{18}$; Thermo Electron Corp., Bellefonte, PA). Fractions were collected per minute and combined fractions were dried in beakers. Then, 50% acetonitrile was used to resuspend the contents in beakers. The antimicrobial activity of each fraction against *L. innocua* ATCC 33090 was tested. Fractions with antimicrobial activity were also re-injected into HPLC to check the purity. Fraction with strongest antimicrobial activity and highest purity was subjected to further MS and NMR analysis.

**MS and NMR analysis.** Purified antimicrobial agent fraction from the previous step was analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis, liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) and NMR analysis.

**MIC determination.** HPLC-purified antimicrobial agent, isolated from OSY-$I_1$, was tested for its antimicrobial activity against selected bacteria indicators (Table 1) according to Clinical and Laboratory Standards Institute (Wikler, 2006). Specifically, stock solutions for this purified antimicrobial agent was prepared in DMSO at a concentration of 3,200 μg/ml. Two-fold serial dilutions were made as working solutions. The MIC experiment was conducted in 96-well microtiter plate including (each well) 178 μl of medium, 20 μl bacterial

indicators (~$2.0 \times 10^4$ CFU/well) and 2 μl of diluted antimicrobial agent. Also, vancomycin (Sigma, St. Louis, MO) and nisin (Aplin and Barrett Ltd. Trowbridge, UK) were prepared the same as we did for the purified antimicrobial agent. The MIC results were examined after incubation at 35 ℃ for 20 hours, and the experiments were done in triplicate.

**Results**

**Isolation and identification of OSY-$I_1$.** In total, 2,500 isolates were isolated from food or environmental samples. Among them, OSY-$I_1$ demonstrated strong (inhibition zone diameter > 3.0 cm) antimicrobial activity. Based on the biochemical test results compared to online database, it showed 99.9% similarity between OSY-$I_1$ and *Brevibacillus laterosporus*. In addition, genetic sequence of 16S rRNA gene (1450 bp) also indicated that isolate OSY-$I_1$ shares high identity (97.7%) with *B. laterosporus*. As a summary, OSY-$I_1$ was identified as *Brevibacillus laterosporus* based on both biochemical and genetic approaches.

**Extraction and purification of the antimicrobial agent isolated from OSY-$I_1$.** As shown in Fig. 1A, the antimicrobial agent was eluted at the retention time of 16.4 min. This active fraction was re-injected into HPLC, and a single isolated peak was observed (Fig. 1B), suggesting that the compound was purified to homogeneity, and this pure agent was designated as brevibacillin.

**MIC determination of brevibacillin.** Purified brevibacillin by using HPLC was used for MIC determination. Selected bacterial indicators are shown in Table 1. Based on Table 1, brevibacillin demonstrated more potency in terms of antimicrobial activity and it also showed similar inhibition ability with vancomycin.

**MS and NMR analysis.** The MS results (Fig.3) showed that the compound has a molecular weight of 1583.0794 and it is consisted with 11 amino acids, without the structural elucidation at both N- and C-terminal. The NMR results indicated 13 amino acids residues and confirmed MS results on many aspects. Both technique, taken together, discovered a novel structure of brevibacillin which is shown in Fig. 2.

In conclusion, the chemical structure of brevibacillin was elucidated as a lipopeptide. It showed strong antimicrobial activity against food spoilage, foodborne illness and antibiotic resistant strains indicating its potency to apply as a food additive and an antibiotic agent.

**Table 1.** MIC (μg/ml) of brevibacillin, vancomycin, and nisin against selected bacteria including food spoilage, foodborne illness and antibiotic-resistant indicators

| **Bacterial strains** | **Brevibacillin** | **Vancomycin** | **Nisin** |
|---|---|---|---|
| **Gram-positives** | | | |
| *Alicyclobacillus acidoterrestris* | 1.0 | <0.5 | <0.5 |
| *A. acidoterrestris* ATCC 49025 | 0.5-1.0 | <0.5 | <0.5 |
| *Bacillus cereus* ATCC 11778 | 2.0-4.0 | 2.0-4.0 | 8.0 |
| *B. cereus* ATCC 14579 | 1.0 | 1.0 | 2.0 |
| *Clostridium difficile* A515 | 4.0-8.0 | 2.0 | 4.0-8.0 |
| *C. difficile* CL148 | 4.0-8.0 | 2.0 | 4.0-8.0 |
| *Enterococcus faecalis* ATCC 29212 | 2.0 | 2.0 | >16.0 |
| *E. faecalis* ATCC 51299, vancomycin resistant | 4.0-8.0 | >16.0 | >16.0 |
| *Lactobacillus plantarum* ATCC 8014 | 1.0 | >16.0 | <0.5 |
| *Lactococcus lactis* ATCC 11454 | 2.0 | <0.5 | >16.0 |
| *Listeria innocua* ATCC 33090 | 1.0-2.0 | 1.0 | 2.0-4.0 |
| *L. monocytogenes* OSY-8578 | 1.0-2.0 | 1.0 | <0.5 |
| *L. monocytogenes* Scott A | 1.0 | 1.0 | 4.0 |
| *Staphylococcus aureus* ATCC 6538 | 1.0-2.0 | 1.0 | 1.0 |
| *S. aureus*, methicillin resistant (MRSA) | 1.0 | 1.0-2.0 | 2.0-4.0 |
| **Gram-negatives** | | | |
| *Escherichia coli* K-12 | >32 | >16 | >16 |
| *E. coli* O157:H7 EDL 933 | 32 | >16 | >16 |
| *Pseudomonas aeruginosa* ATCC 27853 | >32 | >16 | >16 |
| *Salmonella* Typhimurium DT 109 | >32 | >16 | >16 |

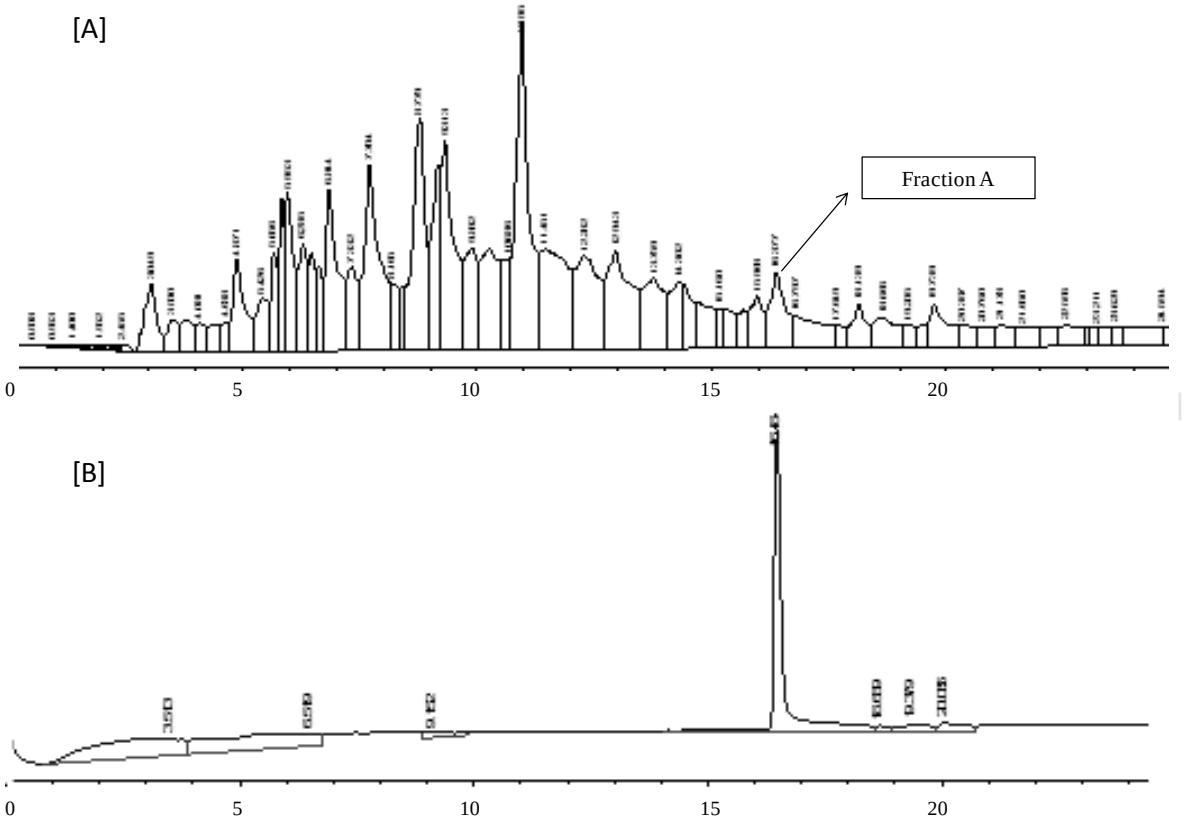


**Figure 1.** [A] HPLC chromatogram of brevibacillin containing crude extract; [B] Purified fraction re-injected into HPLC and showed a single, isolated peak.

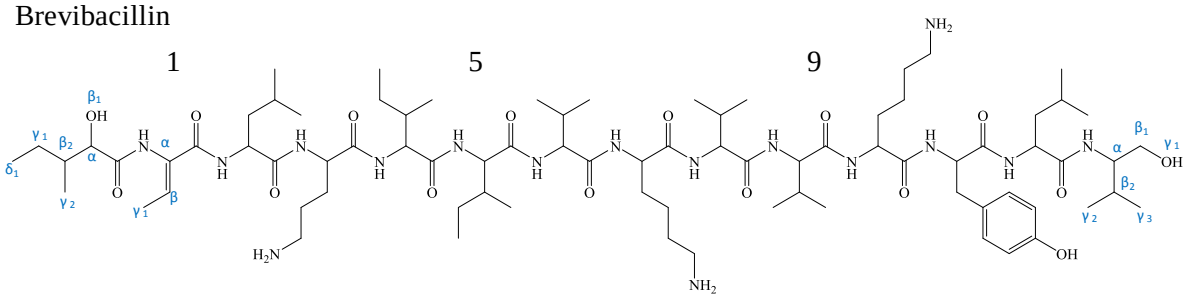


**Figure 2.** Chemical structure of brevibacillin, which includes 13 amino acid residues with a lipid chain attached at N-terminal.

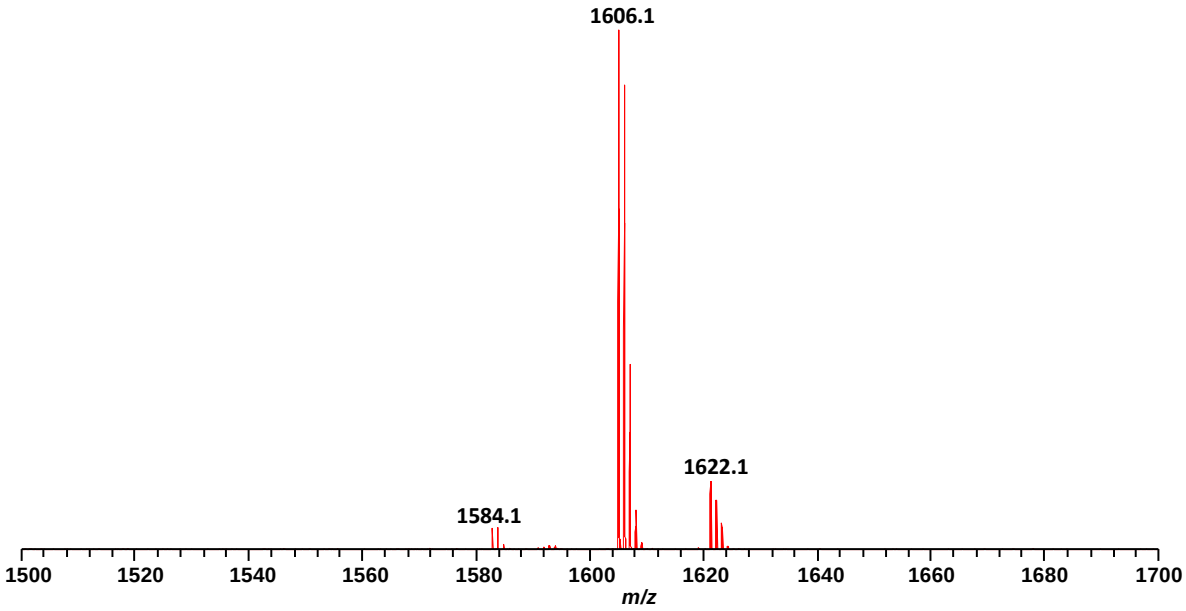


Figure 3. Molecular weight of brevibacillin, as analyzed by MALDI-TOF.

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