Keywords: Anaerobe; Fermentation; Lactobacillus; Antibacterial; Polypeptide

Introduction

Conventional antibiotics are not easily degraded, and residues can accumulate in the environment which promotes the emergence of multi-drug resistant strains [1]. In clinical medicine, the emergence of drug-resistant strains limits the effectiveness of conventional antibiotic therapy, which could be life-threatening to patients if treatment is nonresponsive to available drugs.

In the fuel ethanol fermentation industry, where fermenting microbes are used to convert biomass sugars to fuels and chemicals, bacterial contamination of the fermentors often lead to down time of the production facilities and increased operational cost [2]. To prevent undesired bacteria from growing and competing for nutrients with fermenting microbes, antimicrobial agents have been used in some commercial fermentation tanks. A disadvantage of applying conventional antibiotics is that they do not degrade easily, such that remaining residues may accumulate along the production process and can contribute to the emergence of multi-drug resistant bacterial strains. Recently, bacterial strains with multidrug resistance to virginiamycin and penicillin were reported in dry-grind ethanol plants [3].

New antibacterial agents and control strategies are needed to prevent and control the prevalent occurrence of bacterial infection/contamination and multi-drug resistance and to reduce or replace conventional antibiotics [1]. This is of particular interest for the ethanol industry because the major fermentation byproduct DDGS has been sold as animal feed. Biodegradable agents with bactericidal activities are promising safe alternatives to synthetic antibiotics and need to be explored in preventing and controlling bacterial infections.

In nature, many microorganisms produce various compounds with anti-bacterial properties. One group of these compounds, bacteriocins, consists of bactericidal proteins with a mechanism of action similar to ionophore antibiotics. Bacteriocins have been described as proteinaceous compounds produced by bacteria that have a biologically active protein moiety and bactericidal action which can inhibit or eliminate the growth of sensitive bacterial species [4]. Bacteriocins are often active against species which are closely related to the producer. Their widespread occurrence from complex microbial communities such as the intestinal tract, oral mucosa, or other epithelial surfaces suggests that bacteriocins may have a regulatory role in terms of population dynamics within bacterial ecosystems.

Most bacteriocins have been identified among lactic acid producing bacteria, which include Lactobacillus species, Bifidobacterium species, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, Leuconostoc mesenteroides, Pediococcus acidilactici, Sporolactobacillus inulinus, Streptococcus thermophilus, etc [5]. These species are in wide use throughout the fermented dairy, food and meat processing industries. They are Gram-positive, nonsporulating, catalase-negative organisms devoid of cytochromes. They are anaerobic but are aerotolerant, fastidious, acid-tolerant, and strictly fermentative with...
lactic acid as the major endproduct of sugar fermentation [6]. Their role in the preservation and flavour characteristics of foods has been well documented [7]. Most of the bacteriocins produced by this group are active only against other lactic acid bacteria, but several display antibacterial activity towards more phylogenetically distant Gram-positive bacteria and, under certain conditions, Gram-negative bacteria [8].

The lantibiotic peptide nisin produced by Lactococcus lactis is the best known and well characterized bacteriocin [9-11]. Nisin is desired because it is a biodegradable antibacterial agent and is safe to use as a food preservative in processed dairy products. However, since nisin cannot be synthesized artificially, the only route of production is via fermentation, involving complicated post-translational modifications, thus, it remains an expensive product. To date, pediocin is the only other bacteriocin which has been used as a food preservative in processed dairy products [12].

With zero tolerance for bacterial contamination in food and feed processing, plus generally restricted antibiotic usage, commercial production of new bacteriocins for applications in food, feed and medicine has drawn more attention [13,14]. In reality, there is a need to develop new bacteriocins with a wide range of antibacterial activities, especially against bacteria that are antibiotic resistant.

In this paper, we report the production of a biodegradable polypeptide laparaxin by a novel bacterial isolate Lactobacillus paracasei that shows antibacterial activities against several antibiotic resistant Staphylococcus aureus strains. The biodegradable bacteriocidal agents have potential health benefits to human beings and the environment. Anticipated applications of laparaxin also exist in the biofuel industry to control bacterial contamination during fermentation. Additional studies in fermentation efficiency, substrate utilization, media formulation and scale-up fermentation are needed to demonstrate potential for commercial production.

Materials and Methods

Bacterial strains and growth conditions

Lactobacillus strains [6,15] were maintained on MRS plates (Becton Dickinson, Sparks, MD) under anaerobic conditions (BBL GasPak anaerobic system, Becton Dickinson, Franklin Lakes, New Jersey) and grown in MRS broth at 30°C without shaking. Staphylococcus aureus strains [16,17] were grown in tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD) or in Mueller-Hinton broth containing CaCl₂ (50 mg L-1) (MHBc) (Becton Dickinson, Sparks, MD) at 37°C with shaking at 210 rpm. Listeria monocytogenes strain 10403S [18] and Enterococcus faecalis CK111 [19] were grown in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, MD) at 37°C with shaking at 210 rpm.

For carbon source utilization tests of Lactobacilli, a simplified MRS medium (designated MRSsi) was developed by varying concentrations of several components in MRS [20]. MRSsi contains the following per liter: 5 g of casamino acids, 5 g of peptone, 5 g of yeast extract, 0.5 ml of Tween 80, 0.05 g of MnSO₄•4H₂O, 0.2 g of MgSO₄•7H₂O, 0.1 g of CaCl₂•6H₂O, and 5 g of sodium acetate. MK media contains the following per liter: 10 g beef extract, 5 g tryptone, 5 g yeast extract, 2 g ammonium citrate, 0.05 g of MnSO₄•4H₂O, 0.1 g of MgSO₄•7H₂O, 2 g K₂HPO₄, 20 g of KH₂PO₄ and 5 g sodium acetate. The pH of the media was adjusted to 6.5. Concentrated sugars were autoclaved separately and added prior to use. Growth was monitored by measuring the A600 periodically and fermentation products were analyzed via HPLC as previously described [21].

Production of crude antibacterial polypeptide

The L. paracasei NRRL B-50314 strain was inoculated in 3 ml of MRS broth and incubated overnight. About 2.5 ml of the culture was then transferred to 500 ml MRS in a close capped media bottle and incubated at 30°C for 24 hours. The bacterial cells were removed by centrifugation and the supernatant was filtered using a Nalgene disposable bottle top filter (pore size 0.2 µm) (Nalgene, Rochester, NY). Aliquots of the filtrate were stored at -20°C. This filtered supernatant from culture broth of L. paracasei NRRL B-50314 was used as crude laparaxin for growth inhibition and gel-overlay assays.

Growth inhibition/antimicrobial activity assay

The bacterial strains used as indicator organisms for gel overlay assays are listed in Table 1. A single colony of the desired indicator bacterium was inoculated in 3 ml of broth as specified above and grown overnight at 30°C. Briefly, 50 µl of indicator bacterial cells of late log phase culture were mixed with 8 ml of melted top agar (0.75 % agar in MRS), which had been cooled to 55°C, and poured immediately onto pre-warmed (room temperature) MRS plates.

Meanwhile, individual samples containing 15 µl of crude laparaxin and 15 µl loading dye were denatured at 90°C for 3 min and subjected to SDS PAGE on 8-16% Tris-HCl gels (Ready gels, BioRad Laboratories, Hercules, CA 94547) at 80 volts for 15 min, followed by 130 volts for 60 min. The gels were washed with ddH₂O for 15 min. The single lanes were excised with a clean razor blade and placed onto the above mentioned fresh solidified top agar containing indicator cells. The plates were left in the hood for 30 min, and then incubated at 30°C or 37°C overnight. The width and height of the clearing zones of each indicator strains were recorded. The gel overlay images were acquired using a digital camera (BioDoc-It System, UVP, Inc. Upland, CA).

Antibacterial activities were also measured by inhibition of growth of the indicator strain Lactococcus lactis LM0230 in 96 well plates. Each well contained 260 µl of MRS broth, 5 µl of fresh overnight culture of L. lactis, and 15 µl of crude laparaxin. The mixture was incubated for 5 hours at 30°C without shaking, after which, growth inhibition of the indicator strain was measured spectrophotometrically at 600 nm with a microplate reader (Spectra Max M5, Molecular Devices, Sunnyvale, CA). The antibacterial activity was calculated against a positive control where MRS media was used to replace the crude laparaxin, and presented as the percentage of inhibition based on A600 of the indicator strain relative to control samples.

16S rDNA sequencing

Genomic DNA of L. paracasei NRRL B-50314 was isolated using the Gram-positive DNA purification kit (Epigenetics, Madison, Wisconsin). Genomic PCRs were performed by using 16s rDNA primers [20], and the PCR fragment was sequenced using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit and the ABI Prism 310 DNA sequencer (Perkin-Elmer, Foster City, California). Sequence analyses were performed with the SDSC biology workbench (http://www.sdsc.edu/Research/biology/) and through the National Center for Biotechnology Information, NCBI (http://www.ncbi.nlm.nih.gov/).
Conversion of various sugars to laparaxin and other fermentation products

Fermentations of *L. paracasei* NRRL B-50314 were performed in 2 liter fermentors (Biostat B, B. Braun International, Germany), at constant pH (6.0) controlled using 4M NaOH and 4M phosphoric acid. Fermentations were carried out by 2% inoculation at 30°C with 100 rpm stirring. Samples were taken periodically during the course of fermentation. The concentrations of residual sugars and fermentation products, including lactate, acetate, and ethanol, were measured by HPLC using a 300 mm Aminex HPX-87H column (Bio Rad, Richmond, CA) and a refractive index detector (G1362A, Agilent Technologies, Palo Alto, CA). Samples were run at 65°C and eluted at 0.6 ml min-1 with 5 mM sulfuric acid [20].

Results and Discussion

Isolation and identification of bacterial contaminants

The *L. paracasei* strain was isolated from a laboratory culture tube of *Lactobacillus buchneri* which showed decreased turbidity after 24 hrs compared to other similar *L. Buchneri* cultures. This particular culture tube was initially suspected to be infected by phage, but later attempts to isolate phage particles were unsuccessful. The culture was then plated out on MRS to test for contamination. Single colonies from the MRS plate were washed by vortexing with sterile water, plated out and re-streaked on MRS. Six individual colonies were inoculated in MRS broth and grown overnight. These cultures were centrifuged and supernatant filtered and used for antibacterial activity test. Simply, 50% of filtered supernatant of overnight cultures were mixed with 50% of the indicator *L. buchneri* culture at A600 0.2. Among 6 isolates, one was capable of delaying growth of *L. buchneri*. This isolate was found closely related to *L. Paracasei* subsp. tolerans by sequence analysis of 16S rDNA. The new strain was deposited in the ARS Culture Collection as *L. paracasei* NRRL B-50314.

Bactericidal activities

To further confirm the growth tests, a gel overlay technique was used to test for antibacterial activities in cell-free culture supernatants of NRRL B-50314. A SDS-PAGE gel slice was placed over a freshly seeded lawn of indicator bacterium within a thin layer of top agar across the surface of an agar plate. The growth of bacteria distributed through the top agar produces a homogeneously turbid lawn after overnight incubation except where antibacterial agents are applied. The inhibition of bacterial growth can reduce or eliminate the turbidity of the lawn near the agent, thus, the antibacterial activity is judged by the width of the zone of inhibition around it. Figures 1a and 1b show inhibition of *L. lactis* and *E. faecalis* respectively by crude laparaxin. It is interesting to note that in addition to highly active low molecular weight laparaxin, there are other higher molecular weight proteins (Figure 1a) that can prevent *L. lactis* growth. The strength of the crude laparaxin over different bacterial species including drugresistant pathogens is presented in Table 1. These results indicated that the crude laparaxin exhibited a broad spectrum of inhibition including pathogens *Listeria monocytogenes* 10403S, *Staphylococcus aureus* COL (a MRSA strain), *S. aureus* SH1000, plus several other antibiotic resistant species such the Gram-positive bacterium *E. faecalis* (Table 1).

Next, production of crude laparaxin was assessed over a 24 hour period. Laparaxin is produced in early exponential phase and reaches its highest level at the stationary phase (Figure 2). Since this experiment was performed in a culture bottle without pH control, the final pH dropped to around 4.0. It is interesting to note that lower inhibitory activity was detected in the pH controlled bioreactor at pH 6.0 when compared to that produced in the bioreactor at pH 5.0 (data not shown). The production of laparaxin was also examined using a range of carbon/energy sources including fructose, mannose, lactose, sucrose, maltose, and cellobiose using both MK and MRSsi media. Strain B-50314 grows well in most of these carbohydrates except cellobiose and xylose. With a 2% inoculum, the maximum A600 reached up to 7.0 in 24 hours when fructose, glucose and sucrose were used (data not shown). When the two types of media were compared, cells grown in MRSsi produced more laparaxin than in MK. Of the carbohydrates tested, glucose, fructose and sucrose were the best carbon-sources for laparaxin production (Figure 3, Table 2).

The crude laparaxin was subjected to high temperature (94°C) treatments ranging from 30 min to 120 min. When compared with a control sample stored at 4°C for one week, heat treatment for greater than 60 min decreased inhibitory activity significantly, but did not abolish the inhibitory activity. The growth inhibition activity of crude

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**Figure 1(a):** Gel overlay assay using crude laparaxin as described in Materials & Methods section. 15 μl of crude laparaxin was subjected to SDS PAGE (8-16% Tris-HCl ready gels from BioRad). The gel piece with molecular weight marker was stained with BioRad Coomassie brilliant blue R-250 and de-stained overnight. The marker gel piece was then aligned in parallel with a fresh gel piece that was rinsed with water for 15 min after electrophoresis and overlaid with *Lactococcus lactis* LM 2030.

**Figure 1(b):** *Enterococcus faecalis* CK111 (1B) respectively to determine which band(s) corresponded to antibacterial activity, thus the estimated molecular weight of the laparaxin.
laparaxin remained effective for 30 min of high temperature (94°C) treatment (Figure 4).

Other fermentation products from *L. Paracasei* NRRL B-50314

In addition to the polypeptide laparaxin which was produced and secreted into culture broth, the other major fermentation product of *L. paracasei* NRRL B-50314 is lactate. The strain can produce lactate from various substrates including glucose, fructose, sucrose, lactose, mannose and maltose (Table 2). The strain does not degrade cellobiose and is unable to use xylose, arabinose, and ribose.

**Summary**

In this study, we reported the discovery of antibacterial activity (designated laparaxin) produced and secreted into the culture broth by a novel strain of *L. paracasei* B-50314. The crude laparaxin inhibits growth of several Gram-positive bacterial pathogens, and the antimicrobial effect is due to a specific polypeptide (laparaxin) as indicated by protein gel analyses. The strength of laparaxin activity varies against different indicator strains, and appeared most potent against *S. aureus* 209P, DU4916S MRSA strains and *L. buchneri* B-30929. The laparaxin is temperature stable, and remains active after 30 min of incubation.

**Table 1:** Antibacterial gel overlay summary against various indicator strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Zone width (cm)</th>
<th>Zone height (cm)</th>
<th>Drug-resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. lactis LM 0230</td>
<td>2.10 ± 0.20</td>
<td>1.70 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>L. monocytogenes 10403S</td>
<td>1.45 ± 0.03</td>
<td>1.00 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis CK111</td>
<td>1.30 ± 0.10</td>
<td>0.90 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>S. aureus SH1000</td>
<td>1.40 ± 0.01</td>
<td>1.00 ± 0.00</td>
<td>MSSA</td>
</tr>
<tr>
<td>S. aureus 209P</td>
<td>2.50 ± 0.55</td>
<td>1.20 ± 0.10</td>
<td>MSSA</td>
</tr>
<tr>
<td>S. aureus DU4916S</td>
<td>2.20 ± 0.15</td>
<td>1.40 ± 0.10</td>
<td>MSSA</td>
</tr>
<tr>
<td>S. aureus 1316 P+M-</td>
<td>1.50 ± 0.05</td>
<td>1.10 ± 0.05</td>
<td>MSSA</td>
</tr>
<tr>
<td>S. aureus 1316 P-M</td>
<td>2.00 ± 0.05</td>
<td>1.40 ± 0.20</td>
<td>MSSA</td>
</tr>
<tr>
<td>S. aureus COL</td>
<td>1.50 ± 0.05</td>
<td>1.00 ± 0.10</td>
<td>MRSA</td>
</tr>
<tr>
<td>S. aureus 592S</td>
<td>1.50 ± 0.15</td>
<td>1.10 ± 0.15</td>
<td>Hetero VISA</td>
</tr>
<tr>
<td>S. aureus MM66</td>
<td>1.50 ± 0.15</td>
<td>1.10 ± 0.15</td>
<td>Hetero VISA</td>
</tr>
<tr>
<td>S. aureus 1316 P-M+ V5 MRSA*</td>
<td>1.90 ± 0.35</td>
<td>1.50 ± 0.30</td>
<td>Homo VISA</td>
</tr>
<tr>
<td>S. aureus 1316 P+M+V20 MRSA*</td>
<td>1.40 ± 0.20</td>
<td>1.00 ± 0.00</td>
<td>Homo VISA</td>
</tr>
</tbody>
</table>


**Table 2:** HPLC analyses of the fermentation products by *L. paracasei* NRRL B-50314 from various substrates.

<table>
<thead>
<tr>
<th>Substrate numbers corresponding to Figure 3</th>
<th>Culture Media</th>
<th>Fermentation products (g/100mls or %)</th>
<th>Lactate</th>
<th>Acetate*</th>
<th>Ethanol*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2% Mannose</td>
<td>1.40</td>
<td>0.35</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2% Lactose</td>
<td>1.42</td>
<td>0.37</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2% Fructose</td>
<td>1.86</td>
<td>0.34</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2% Glucose</td>
<td>1.67</td>
<td>0.66</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2% Glucose MRsai</td>
<td>1.78</td>
<td>0.45</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1% Sucrose MRsai</td>
<td>1.05</td>
<td>0.45</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1% Cellobiose MRsai</td>
<td>0.37</td>
<td>0.46</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

*Both of the original MRsai and MK media contain 0.5% (5 g per liter) acetate. Only negligible trace of ethanol was detected.

**Figure 2:** Growth analysis of *L. paracasei* NRRL B-50314 as measured by change of optical density at 600 nm over 32 hrs period (*). The activity of crude laparaxin produced by *L. paracasei* NRRL B-50314 at each of the corresponding time points were determined by growth inhibition assay against *L. lactis* LM 2030 as described in Materials & Methods section. The % inhibition activity (%) was calculated as the average of three experiments relative to control *L. lactis* cultures in which crude laparaxin was replaced with MRS.

**Figure 3:** Effects of different media and carbon sources on the production of crude laparaxin by *L. paracasei* NRRL B-50314. Specifically, carbon sources of MK 2% mannose (1), MK 2% lactose (2), MK 2% fructose (3), MK 2% glucose (4), MRsai 2% glucose (5), MRsai 1% sucrose (6), and MRsai 1% cellobiose (7) were shown. Components of MK and MRsai are described in Materials & Methods. The antibacterial assays were performed in 96 well plates against *L. lactis* LM 2030 with the amounts of each substrate indicated as % (g/100 ml). The substrate percentages depicted are an average of three experiments.

**Figure 4:** Effects of high temperature treatments on antibacterial activities of the crude laparaxin. Samples were placed in a heat block at 94°C and taken out after 30, 60, 90 and 120 minutes incubation. Growth inhibition assays were performed in 96 well plates against *L. lactis* LM 2030 and the average of three experiments were presented here.

at 94°C. Of the six carbon mono- and disaccharides tested, glucose, fructose and sucrose are the best growth substrates for laparaxin production.

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References


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