Published in final edited form as:
J Appl Microbiol. 2008 April ; 104(4): 1067–1074.

Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product-derived *Bacillus amyloliquefaciens*

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Abstract

Aims—To purify and characterize an antimicrobial protein (bacteriocin) isolated from the dairy product-derived *Bacillus amyloliquefaciens.*

Methods and Results—An unknown bacterial species cultured from the Yogu Farm™ probiotic dairy beverage was identified through 16S ribosomal RNA analysis as *B. amyloliquefaciens*, a phylogenetically close relative of *Bacillus subtilis.* The cell-free supernatant (CFS) of overnight cultures was active against *Listeria monocytogenes* and also against clinical isolates of *Gardnerella vaginalis* and *Streptococcus agalactiae.* At the same time, several isolates of vaginal probiotic *Lactobacilli* were resistant to the CFS. The nature of the compound causing inhibitory activity was confirmed as proteinaceous by enzymatic digestion. The protein was isolated using ammonium sulfate precipitation, and further purified via column chromatography. PCR analysis was conducted to determine relatedness to other bacteriocins produced by *Bacillus* spp.

Conclusion—The antimicrobial protein isolated from *B. amyloliquefaciens* was shown to be subtilosin, a bacteriocin previously reported as produced only by *B. subtilis*.

Significance and Impact of the Study—This is the first report of intra-species horizontal gene transfer for subtilosin and the first fully characterized bacteriocin isolated from *B. amyloliquefaciens*. Finally, this is the first report on subtilosin's activity against bacterial vaginosis-associated pathogens.

Keywords

antimicrobial; *Bacillus amyloliquefaciens*; *Bacillus subtilis*; bacteriocin; subtilosin

Introduction

Bacteriocins are ribosomally synthesized proteins produced by a diverse group of micro-organisms that elicit bactericidal activity, usually against closely related species (Klaenhammer 1993; Guinane *et al.* 2005; Drider *et al.* 2006; Nagao *et al.* 2006). *Bacillus subtilis* is a known producer of many antibiotic and antimicrobial compounds, including the bacteriocin subtilosin A (subtilosin) (Stein 2005). Subtilosin A was originally isolated by Babasaki *et al.* (1985) from the wild strain *B. subtilis* 168, and an early, incomplete amino acid sequence was reported. A complete amino acid sequence was later published by Zheng *et al.* (1999), and further elucidation was provided by Marx *et al.* (2001) using ^{1}H-NMR to produce a 3-D image of the molecule's structure. It was determined to be a circular molecule of 35 amino acids with a very

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unique post-translational structure, namely three sulfur cross-links between cysteine and the α-carbon of two phenylalanines and a threonine residue. As this configuration is unparalleled in other antimicrobial peptides, it is speculated that subtilosin may belong to a unique class of bacteriocins (Kawulka *et al.* 2004).

Horizontal gene transfer (HGT) is a mechanism employed by bacteria as a means of acquiring new genetic properties. Although it was once difficult to establish instances of HGT, genetic analysis now provides unmistakable supportive evidence. The evolutionary modification of traits is typically a slow and lengthy process defined by point mutations that inactivate or activate new regions of genes. In comparison, HGT can rapidly change whole features of a species for generations to come. In order to occur, bacteria must possess a means of acquiring the new information from a neighbouring species, e.g. competency (Ochman *et al.* 2000). In both *B. subtilis* and *Streptococcus mutans* competency has been linked to bacteriocin production, suggesting that this mechanism may be prevalent among organisms in multi-species environments (D'Souza *et al.* 2005; Hamoen *et al.* 2005; Kreth *et al.* 2005).

There have been several documented cases of HGT involving bacteriocins, specifically those involving the well-characterized and utilized class I bacteriocin nisin. Primarily produced by *Lactococcus lactis* subsp. *Lactis* (Klaenhammer 1993), genes encoding for the production of and resistance to this small protein have also been isolated from several other *L. lactis* subspecies (Gireesh *et al.* 1992), as well as *Leuconostoc dextranicum* (Tsai and Sandine 1987). Muriana and Klaenhammer (1992) also reported on the conjugal transfer of bacteriocin production determinants in *Lactobacillus acidophilus* 88. While many strains of *B. subtilis* have been identified as subtilosin producers (Stein *et al.* 2004), there are no documented cases of the presence of its structural and functional genes, or reported production of it, in another species. Here, we describe the production, purification, antimicrobial activity and genetic identification of subtilosin from a *Bacillus amyloliquefaciens* culture.

Materials and methods

Bacterial strains, growth conditions and culture media

Bacillus amyloliquefaciens was isolated from the yogurt-flavoured cultured beverage Yogu Farm™ (JSL Foods, Los Angeles, CA, USA) purchased from Hong Kong Market, New Brunswick, NJ, USA, by aliquoting 1 ml of the product into 20 ml of MRS broth (Difco™, Detroit, MI, USA). The culture was incubated for 48 h at 37°C in 5% CO_2 atmosphere without agitation. Inoculated plates were also incubated in the same conditions. Samples of the liquid culture were examined with phase microscopy to visualize basic cell characteristics. Culture samples were sent to the Laboratory for Molecular Genetics (Cornell University, Ithaca, NY, USA) for ribotyping and to Accugenix (Newark, DE, USA) for 16S ribosomal RNA (rRNA) analysis to confirm the identity of the unknown organism. *Micrococcus luteus* ATCC 10420, *Listeria monocytogenes* Scott A and *Salmonella* Typhimurium ATCC 14028-1s were grown in Tryptic Soy Broth supplemented with 0.6% Yeast Extract (Difco) at 30°C under aerobic conditions. *Pediococcus pentosaceus* ATCC 43200 was cultivated in MRS broth at 37°C for 24 h under aerobic conditions. *Gardnerella vaginalis* ATCC 14018 was grown on HBT agar (BD, Franklin Lakes, NJ, USA), while *Streptococcus agalactiae* (Group B Streptococcus) was grown on Columbia agar with 5% Sheep Blood (BD). Both organisms were incubated at 36° C in 5% CO_2 atmosphere without agitation. The indicator strains used in well diffusion assays were obtained from either ATCC collections or as clinical isolates from the Rush Presbyterian Medical Center in Chicago, IL, USA (Table 1).

Sample preparation

Cell-free supernatant (CFS) harvested from MRS broths was incubated for 48 h at 37°C in 5% CO_2 atmosphere (until approx. 10^6 CFU ml^{-1}). Cells were removed from the culture by centrifugation (Hermle Z400K; LabNet, Woodbridge, NJ, USA) for 25 min at 4500 ***g*** and 4° C. Supernatants were filter-sterilized using 0.45 μm microfilters (Fisher, Pittsburgh, PA, USA).

Assay of antimicrobial activity

Well diffusion inhibition assays were conducted as described by Cintas *et al.* (1995) with the following modifications. The efficacy of the *B. amyloliquefaciens* product at inhibiting the growth of various micro-organisms was tested using CFS against MRS broth as a negative control and nisin (10 mg ml^{-1}) [Sigma, 2.5% bacteriocin preparation (10^6 IU g^{-1}) dissolved in ddH_2O] as a positive control. The indicator organism was grown overnight according to its specific growth requirements, with *M. luteus* used as a standard based on its known sensitivity to bacteriocins (Pongtharangkul and Demirci 2004). Soft agar was made by adding 0.7% agar to either TGY or MRS; solid base plates were dried in a sterile hood for approx. 90 min prior to use in order to remove any extraneous moisture. To create an overlay, the indicator organism was added to the soft agar in a ratio of 100 μl of bacterial culture per 10 ml soft agar (*c.* 10^6 CFU ml^{-1}). From this mixture, 4 ml was overlaid onto each base plate and allowed to completely solidify. Pasteur pipettes were used to create 5-mm wells in the overlaid base plates. These wells were then allowed to dry for approx. 30 min. Then, 50 μl of each sample was added to the wells and allowed to freely diffuse for 45–60 min. All plates were then incubated overnight at the optimal growing conditions for the indicator organism (Table 1). The procedure for testing activity against the clinical isolates varied slightly from the previously described method. The indicator organism was inoculated as a lawn using a sterile swab, and after air-drying for 5 min, 17-mm wells were punched into the agar using a sterile glass test tube and 400 μl of CFS was added. The plates were kept at room temperature for 2 h to allow for absorption of the supernatant, and then incubated overnight at 36°C with 5% CO_2 atmosphere.

Determination of lactic acid concentrations

Lactic acid concentrations in the CFS were determined using a D-lactic acid/L-lactic acid test kit and according to the manufacturer's protocol (Roche Boehringer, Mannheim, Germany). After completing the steps of the protocol, the gathered data were applied to the provided equations in order to accurately calculate the quantities of each acid form in the sample.

Enzymatic digestion to confirm proteinaceous nature of antimicrobial compound

The CFS was exposed to seven different enzymes (Sigma; Table 2) overnight to determine the type of compound causing bacterial growth inhibition. Aliquots (250 μl) of the CFS were combined with equal volumes of the enzymes and the pH of the mixture and the incubation temperature were adjusted to those optimal for enzymatic activity. Two controls were used: (i) the enzyme mixed with sterile MRS media, and (ii) the CFS and enzyme diluent (Table 2). After 24 h the pH of all samples was readjusted to ~6 to attain maximum antimicrobial activity. Well diffusion assays were conducted in triplicate against the indicator *M. luteus.*

Protein visualization

SDS-PAGE was conducted using a Tris-Tricine gel made in a Bio-Rad casting apparatus (Bio-Rad, Hercules, CA, USA) according to the protocol given by Schägger and Von Jagow (1987). The gels were loaded with either 20 μl of marker or 200 μl of sample [1: 1 sample + loading buffer (Bio-Rad)]. Nisin (10^6 IU g^{-1}, 2 mg ml^{-1}) was used as the positive control. The procedure was conducted in 0.2 mol l^{-1} Tris-base anode running buffer (pH 8.9) and 0.1 mol l^{-1} Tris/0.1 mol l^{-1} Tricine/0.1% SDS cathode running buffer (pH 8.25) in a Mini-Protean 3 (Bio-Rad) chamber with Power-Pac 300 power source (Bio-Rad).

Upon completion of electrophoresis, the gel was cut into identical halves; one half was treated for the overlay process while the other was used in the staining procedure. The overlay gel was fixed for 2 h in 100 ml of 10% acetic acid/20% isopropanol buffer, rinsed three times over 2 h in 100 ml ddH_2O and stored overnight in ddH_2O at 4°C (all steps occurred under rotation). The following day, it was laid onto a dried enriched TSA plate and overlaid with *M. luteus.* The staining gel was processed according to the manufacturer's Silver Stain protocol (Bio-Rad).

Protein purification

Ammonium sulfate precipitation—Using a stock overnight culture, *B. amyloliquefaciens* was inoculated (*c.* 10^6 CFU ml^{-1}) and grown in 500 ml MRS under normal conditions. Cells were removed by centrifugation for 25 min at 12 120 ***g***. The CFS was filter-sterilized as previously described. A nomogram (Dixon 1953) was used to calculate the amount of solid ammonium sulfate needed to achieve 30% saturation, which was added to the solution incubated at 4°C overnight while stirring. The following day, the precipitate was gathered by centrifugation as described above and re-dissolved in 20 ml ddH_2O. Activity of both the precipitate and the supernatant were tested in a well diffusion assay against *M. luteus.* The precipitate was used to conduct all further experiments and is designated as the 'sample'.

Column chromatography—Further purification of the 30% ammonium sulfate precipitate was achieved with Sep-Pak® Light C18 Cartridges (Waters, Milford, MA, USA) to separate the protein of interest based on an assumed hydrophobic nature. In each instance, 0.5 ml of liquid was passed through the column at a flow rate of 0.2 ml min^{-1}. The cartridge was initially rinsed with 0.5 ml 100% methanol and equilibrated by four 0.5 ml washes with ddH_2O to remove any traces of the methanol. Following the water washes, the sample was loaded onto the cartridge and the flow-through was collected. This was followed by another four 0.5 ml washes with ddH_2O, with each fraction collected individually. Immediately after the water washes, the column was washed sequentially with 1 ml of 50%, 70%, 90% and 100% methanol, and individual 0.5 ml fractions were collected. Antimicrobial activity was confirmed by the well diffusion assay.

Effect of temperature and pH on antimicrobial activity

Heat shock—The ability of the compound to retain activity under elevated temperatures was tested by incubating the sample at a given temperature for 0–60 min. After each time point 200 μl was aliquoted and used to create two-fold serial dilutions in ddH_2O. Each dilution was used in a well diffusion assay; the reciprocal value of the lowest dilution that maintained activity is considered the protein concentration in arbitrary units (AU) ml^{-1}.

pH stress—The level of antimicrobial activity of the sample was tested at varying pH levels. The pH of the solution was adjusted to fall within the range of 2–10 using either 3 mol l^{-1} HCl or NaOH. The samples were incubated at room temperature for 1 min before conducting a well diffusion assay against *M. luteus*.

Genetic analysis

DNA extraction—DNA was extracted from overnight cultures of *B. amyloliquefaciens* and *B. subtilis* ATCC 6633 using the Promega Wizard SV Genomic DNA Kit (Promega Corp., Madison, WI, USA) with the following modifications. Cells were harvested from the culture (2 × 1.5 ml) in a microfuge tube at 13 000 ***g*** for 3 min and resuspended in 382 μl 0.5 mol l^{-1} EDTA (pH 8.0). To this, 100 μl of lysozyme (20 mg ml^{-1}), 10 μl proteinase K (20 mg ml^{-1}) and 8 μl mutanolysin (2.5 U μl^{-1}) was added. The mixture was incubated for 60 min at 37°C, following which 200 μl of nuclei lysis solution and 5 μl RNase A were added, and incubated

for 20 min at 65°C. Two hundred and fifty microlitres of lysis buffer was immediately added, and DNA was subsequently purified using the provided spin columns according to the manufacturer's specifications and eluted in 100 μl nuclease-free water.

PCR testing—Polymerase chain reactions (PCRs) were performed to assess the relatedness between the bacteriocin produced by *B. amyloliquefaciens* and the *B. subtilis* products subtilin and subtilosin. Primers (listed in Table 3) were designed using the *B. subtilis* genome (GenBank accession no. AJ430547) to specifically recognize the functional genes of subtilin (*spaS*) and subtilosin (*sboA*). Genomic DNA from *B. amyloliquefaciens* and *B. subtilis* ATCC 6633 was added to a master mix consisting of each primer, nucleotides, buffer and HotMaster Taq (Eppendorf, Hamburg, Germany). PCR was conducted using an Applied Biosystems GeneAmp PCR System 2400 apparatus (Applied Biosystems, Foster City, CA, USA) under the following parameters: denaturation for 30 s at 94°C, annealing for 30 s at 55°C (*spaS*) or 50°C (*sboA*), and elongation for 1 min at 65°C for a total of 30 cycles. PCR products were sequenced using ABI Prism 3730*xl* DNA analysers (GeneWiz, Inc., South Plainfield, NJ, USA), and the resulting sequences were analysed using the Vector NTI software suite of programs (Invitrogen, Carlsbad, CA, USA). The sequence obtained for *B. amyloliquefaciens* has been submitted to GenBank under the accession no. EU105395.

Results

Characterization of unknown isolate

While the Yogu Farm™ beverage was purported to contain *Lactobacillus* cultures, *B. amyloliquefaciens* was the only organism recovered from four individual lots of the product. Phase microscopy of each sample of bacterial growth revealed a single organism that was a very motile endospore-producing bacillus. On solid agar, the colonies tended to spread quickly into lawn formation, with an extremely wrinkled texture. The organism appeared to secrete a thick, opaque slime from the colonies, which was later revealed to be a byproduct of the starch-hydrolysing enzyme amylase. Ribotyping and 16S rRNA analyses determined the bacterium to be *B. amyloliquefaciens*, a closely related species to *B. subtilis.*

Range of antimicrobial activity

The CFS of a *B. amyloliquefaciens* culture was determined to have antimicrobial activity against a wide range of bacterial species, including the pathogens *L. monocytogenes*, *G. vaginalis* and *S. agalactiae.* There was no activity against several strains of vaginal probiotic *Lactobacilli* also gathered from the clinical setting (Table 1).

Determination of lactic acid concentrations

Using equations provided by the manufacturer's protocol, it was determined that *B. amyloliquefaciens* produced very low levels of both D- and L-lactic acid in three separately conducted assays. Calculations revealed that there was an average of 0.17 g l^{-1} D-lactic acid per sample, a value equal to that of the tested blank. The average concentration of L-lactic acid rose to 2.22 g l^{-1}, which was slightly higher than the blank's concentration of 0.15 g l^{-1} (Table 4). The very low basal concentrations of both forms of lactic acid suggest they do not play a significant role in microbial inhibition, and that all detected activity may be attributed to the bacteriocin.

Effect of enzyme digestion, temperature and pH on antimicrobial activity

Inhibition assays revealed that activity was completely lost in the presence of pepsin and proteinase K, and significantly decreased by trypsin and chymotrypsin, confirming the proteinaceous nature of the compound (Table 2). Exposure to increasingly high temperatures

had no apparent effect on the protein, with activity still present (64 AU) after the sample had been heated for 60 min at 100°C (Table 5). There was also no reduction in activity at any of the pH values ranging from 2 to 10, despite the fact that the pH of the CFS was typically neutral (~6.5) (data not shown).

Protein purification

Ammonium sulfate precipitation—The protein was fully precipitated out of solution at 30% ammonium sulfate concentration, and the presence of the bacteriocin was confirmed on SDS-PAGE gels, with a large zone of inhibition in the overlay portion corresponding to the known size of subtilosin (data not shown).

Column chromatography—Inhibition assays indicated that the protein was solely and completely eluted from the columns by 90% methanol. They also confirmed activity was wholly due to the anti-microbial peptide and not background activity from the methanol.

Genetic analysis

PCR analysis showed *B. amyloliquefaciens* to be negative for the functional gene-encoding subtilin (*spaS*), but positive for the functional gene-encoding subtilosin (*sboA*) (Stein *et al.* 2004). The DNA sequence of the PCR product amplified from *B. amyloliquefaciens* was compared to that from *B. subtilis* ATCC 6633, and was shown to be 91.7% identical. There were only 3 bp changes in *sboA*, none of which affected the amino acid sequence of the protein. A homologue of *sboX* (95% identical), and a gene that putatively encodes a bacteriocin-like substance and overlaps *sboA*, was also identified. The gene-encoding YwiA (*albA*) is downstream of the gene-encoding SboA, and is believed to have a role in the post-translational modifications of subtilosin (Stein *et al.* 2004). Due to the overwhelming similarity of the two gene products, the sequence preceding the gene and the intergenic sequence were compared, and found to be 95.6% and 85% similar, respectively.

Discussion

Bacillus amyloliquefaciens was isolated from a fermented dairy beverage purported to contain *Lactobacillus* cultures. The bacteria produced a compound, later determined to be a protein, which possessed potent antimicrobial activity against such pathogens as *L. monocytogenes* and *S. agalactiae.* Of particular importance is the fact that while activity was strong against the vaginal pathogen *G. vaginalis*, there was no activity against the clinically isolated probiotic organisms tested.

The bacteriocin was shown to be remarkably stable under extreme temperature and pH stresses, with full activity retained after an hour at 100°C and across the pH range of 2–10. These observations increase the likelihood of this compound being considered for food preservation and personal care applications, as it can be adapted to, and function in, a variety of harsh environments.

Through genetic analysis, the unknown protein was later discovered to be identical to the bacteriocin subtilosin, which is produced by *B. subtilis.* Although there are well-documented cases of subtilosin production by several *B. subtilis* subspecies and the closely related species *B. atrophaeus* (Stein *et al.* 2004), this is the first report of production originating in *B. amyloliquefaciens.* Recent advents in genetic analysis have allowed for a comparison of the 16S rRNA regions and 16S–23S internal transcribed spacer regions of the two species, demonstrating their extreme similarity (Xu and Côté 2003). However, *B. amyloliquefaciens* is generally accepted as a separate and individual species based on its higher GC content (~44% *vs* ~42%), ability to grow in 10% NaCl, and increased production of α-amylase (Welker and

Campbell 1967). The possibility that *B. amyloliquefaciens* is a closely related, yet diverged species, is supported by the research of Hoa *et al.* (2002). They utilized transcriptional profiling to reveal that the *B. subtilis* Rok protein (encoded by *rok*) had an orthologue in *B. amyloliquefaciens*, but not in other bacilli or Gram-positive, spore-forming bacteria, and suggested that *rok* could have been introduced into the *B. amyloliquefaciens* genome by HGT (Albano *et al.* 2005).

With the recent surge in bacteria developing antibiotic resistance, there has been a marked increase in the level of attention given to bacteriocins. These proteins are considered ideal candidates for food preservation and personal care applications because the range of their activity is limited to only closely related species. Therefore, while they may target a specific pathogen, they would theoretically have no harmful effects on humans and their normal microbiota. One of the most studied bacteriocins, nisin, is the only bacteriocin given Generally Recognized as Safe status by the U.S. Food and Drug Administration, leading to its use as a mainstream preservative. Despite the potential of nisin, organisms such as *L. monocytogenes* have developed complex mechanisms rendering them resistant to such treatments (Crandall and Montville 1994; Gravesen *et al.* 2001; Bonnet *et al.* 2006). Subtilosin has a proven track record of efficacy against *L. monocytogenes*, as indicated by this study and others (Zheng *et al.* 1999; Stein *et al.* 2004). This posits it as an attractive option that should be investigated by the food industry.

Very recently, Shelburne *et al.* (2007) conducted the most comprehensive study to date on the spectrum of antimicrobial activity of subtilosin. Their findings suggest that while the bacteriocin has the ability to act on a wide range of organisms, its inefficacy against capsulated organisms limits its practical value. The authors greatly diminish the importance of subtilosin as an antimicrobial agent; this, however, is contradicted by our results indicating its usefulness against vaginal pathogens such as *G. vaginalis* or *S. agalactiae*. The ability of subtilosin to inhibit these species without damaging the healthy microbiota of that niche leads us to propose it may be an effective and safe way to treat infections that, in the case of *G. vaginalis*, have proven adept at developing antibiotic resistance to the drugs of choice for treating bacterial vaginosis (McLean and McGroarty 1996). Our future research with subtilosin will investigate its safety and toxicity to vaginal tissues as a means of determining its potential in practical care applications.

Acknowledgements

This study was supported by the NIH grant ‘Natural antimicrobials against bacterial vaginosis’ NCCAM NIH R21AT002897-01.

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Table 1

Growth conditions and subtilosin sensitivity of indicator organisms

Organism	Growth media	Growth temperature(°C)	Range of antimicrobial activity (mm)
Micrococcus luteus ATCC 10420	TGY	30	25 ± 1.5
Listeria monocytogenes Scott A	TGY	30	18 ± 0.5
Pediococcus pentosaceus ATCC 43200	MRS	37	0
Salmonella Typhimurium ATCC 14028-1s	TGY	30	0
Lactobacillus L711 (clinical isolate)	MRS	30	0
Lactobacillus L735 (clinical isolate)	MRS	30	0
Lactobacillus L807 (clinical isolate)	MRS	30	0
Gardnerella vaginalis ATCC 14018	HBT	36	22
Gardnerella vaginalis (clinical isolate)	HBT	36	28
Streptococcus agalactiae (clinical isolate)	Columbia agar with 5% Sheep Blood	36	20

Table 2

Effect of enzymatic digestion on antimicrobial activity

Enzyme	Enzyme diluent	Enzyme concentration(mg ml^{-1})	Zone of inhibition(mm)*
Catalase	50 mmol l^{-1} KPi (pH 7)	10	10
Pepsin	10 mmol l^{-1} HCl	10	0
Proteinase K	ddH_2O	10	0
Trypsin	1 mmol l^{-1} HCl	20	5
Chymotrypsin	1 mmol l^{-1} HCl	10	6
Lipase	ddH_2O	20	9
Protease	ddH_2O	10	7

* Average zone of inhibition for undigested cell-free supernatant was 10 mm.

Table 3

Specific primers for the functional genes of subtilin and subtilosin

Bacteriocin	Primer	Primer sequence (5′ to 3′)	Reference
Subtilin	spaSFwd	CAAAGTTCGATGATTTCGATTTGGATGT	Klein *et al.* (1992)
	spaSRev	GCAGTTACAAGTTAGTGTTTGAAGGAA	
Subtilosin	sboAFwd	CGCGCAAGTAGTCGATTTCTAACA	Stein *et al.* (2004)
	sboARev	CGCGCAAGTAGTCGATTTCTAACA	

Table 4

Concentrations of D- and L-lactic acid in cell-free supernatant

Sample	ΔA_D	[D-Lactic acid] (g l^{-1})	ΔA_L	[L-Lactic acid] (g l^{-1})
Blank	0.053	0.17	0.047	0.15
Sample 1	0.046	0.15	0.692	2.24
Sample 2	0.054	0.17	0.679	2.19
Sample 3	0.059	0.19	0.686	2.22

Table 5

Effect of temperature stress on antimicrobial activity

Temperature (°C)	Exposure time (min)	Highest active dilution (AU)
60	5	64
	60	64
80	5	64
	60	64
100	5	64
	60	64