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ORIGINAL ARTICLE

Antimicrobial activity of a UV-stable bacteriocin-like inhibitory substance (BLIS) produced by *Enterococcus faecium* strain DSH20 against vancomycin-resistant *Enterococcus* (VRE) strains

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Background/purpose: The narrow spectrum of action of most bacteriocins is an important limitation for their application as antimicrobial agents. The current study describes a novel bacteriocin-like inhibitory substance (BLIS) that display extended spectrum antimicrobial activity against vancomycin-resistant *Enterococcus* (VRE) strains.
Methods: Acquired resistance profiles of *Enterococcus* isolates determined based on the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) definition as multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug resistant (PDR). BLIS activity of *Enterococcus* isolates was investigated against *Enterococcus faecalis* (*E. faecalis*) ATCC 29212 as the indicator strain and clinical isolates including VRE, methicillin resistant *Staphylococcus aureus* (MRSA) and Gram-negative bacteria containing *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella*, *Acinetobacter*, and *Escherichia coli* (*E. coli*).

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Results: Among 273 *Enterococcus* isolates, 27 and 2 VRE isolates, respectively, were XDR and PDR and eight isolates had BLIS activity against the indicator strain. One of these isolates, identified as *E. faecium* strain DSH20 based on its phenotypical and biochemical properties, as well as its 16S rRNA gene sequence, had potent BLIS production against all 29 VRE strains, but had no activity against MRSA, *P. aeruginosa*, *Klebsiella*, *Acinetobacter*, and *E. coli* strains. It was heat stable up to 121°C for 15 minutes (autoclave condition), active within the pH range of 3—9 and had UV stability, but its activity disappeared by treatment with proteinase K, pepsin, and trypsin, demonstrating its proteinaceous nature. It was designated as an approximately 35 kDa peptide using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method.
Conclusion: This peptide is a potential agent for use as an alternative antibacterial agent for the treatment of drug-resistant strains of VRE infection.

Introduction

The appearance and spread of antibiotic-resistant bacteria are serious problems in public health worldwide.[1,2] For example, pandrug-resistant (PDR) isolates were defined as non-susceptible to all agents in all antimicrobial categories based on the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) definitions.[3] Therefore, these strains that are resistant to all tested antibiotic agents lead to curative failure of therapy and are serious threats to the patient's safety. Vancomycin-resistant *Enterococcus* (VRE) has developed a resistance to most commonly used antibiotics and there are currently very few antibiotics that can be used to treat these infections. New strategies for controlling these bacteria are urgently needed and the discovery of new antimicrobial agents, such as bacteriocin and bacteriocin-like inhibitory substance (BLIS), may help in keeping down the development of these infections.[1] Bacteriocins, secondary metabolites produced by various microorganisms, have bactericidal activity directed against species that are usually closely related to the producer microorganisms.[4—6] One of the critical limitations to the use of bacteriocins as antimicrobial and biopreservative agents, is their narrow spectrum of action.[7] The objective of this study was the isolation and characterization of broad spectrum BLIS active against PDR strains of VRE.

Materials and methods

Characterization and identification of bacteria from clinical specimens

Bacteria recovered from clinical specimens were identified by standard biochemical methods.[8] The samples were cultured on blood agar (purchased from Himedia Company, Mumbai, Vadhani Industrial Estate, India), the plates were incubated at 35°C for 24 hours and the pure isolates were characterized and identified according to Gram stains and biochemical tests, such as catalase reaction, presence of pyrrolidonyl arylamidase (PYR), growth on bile aesculin agar and 6.5% NaCl media.

Antimicrobial categories and agents used to define acquired resistance profiles in *Enterococcus* sp.

To define the acquired resistance profiles of *Enterococcus* isolates, antimicrobial categories and agents were used as shown in Table 1, according to ECDC and CDC definition.[3] A multidrug-resistant (MDR) isolate was defined as being non-susceptible to at least one agent in three or more antimicrobial categories. An extensively drug-resistant (XDR) isolate was defined as being non-susceptible to at least one agent in all but two or fewer antimicrobial categories (i.e., bacterial isolates remain susceptible to only one or two categories). Non-susceptibility to all agents in all antimicrobial categories (i.e., no agents tested as susceptible) was defined as PDR *Enterococcus*. Thus, a bacterial isolate that is characterized as XDR will also be characterized as MDR and similarly, an XDR bacterial isolate

Table 1 Antimicrobial categories and agents used to define pandrug resistant (PDR), extensively drug-resistant (XDR) and multidrug-resistant (MDR) in *Enterococcus* spp[3]

Antimicrobial category	Antimicrobial agent
Aminoglycosides (except streptomycin)	Gentamicin (high level)
Streptomycin	Streptomycin (high level)
Carbapenems (except for *Enterococcus faecium*)	Imipenem Meropenem Doripenem
Fluoroquinolones	Ciprofloxacin Levofloxacin Moxifloxacin
Glycopeptides	Vancomycin Teicoplanin
Glycylcyclines	Tigecycline
Lipopeptides	Daptomycin
Oxazolidinones	Linezolid
Penicillins	Ampicillin
Streptogramins (except for *Enterococcus faecalis*)	Quinupristin-dalfopristin
Tetracycline	Doxycycline Minocycline

would have to be further defined as PDR. For example, an isolate that is just sensitive to gentamicin (belongs to the aminoglycosides category), imipenem (belongs to the carbapenems category) and linezolid (belong to the oxazolidinones category) but is resistant to other agents, is a PDR strain. An isolate that is just sensitive to linezolid and is resistant to all other agents, is an XDR isolate (and also PDR) and an isolate that is resistant to all agents mentioned in Table 1, is a PDR isolate (and also MDR and XDR).[3] An antibiotic sensitivity pattern was determined by the Kirby Bauer's disc diffusion method on Mueller-Hinton agar. The MIC method, based on the E-test method, for confirmation of vancomycin resistance in VRE isolates was used. All antimicrobial disks used for susceptibility testing were obtained from (BD: Becton, Dickinson Company, USA). *Enterococcus faecalis* (*E. faecalis*) ATCC 29212 and *E. faecium* ATCC 6569 were used for quality control, as recommended by Clinical and Laboratory Standards Institute (CLSI). The plates were incubated at 35°C for 18 hours. The degree of isolate susceptibility to each antibiotic was interpreted as sensitive (S), intermediate resistant (I) or resistant (R) by measuring the zone diameter of inhibition.

Antimicrobial agents screening

For the antimicrobial potency production assay, a qualitative bioassay was performed using the spot-on-lawn method as follows: a medium consisting of trypticase soy broth supplemented with 0.6% yeast extract (TSBYE) without glucose and 1.5% agar prepared and spotted with 2 μL of isolated strains, was incubated overnight at 30°C for 24 hours. The incubated agar plates were overlaid with a soft-agar medium seeded with 10^7/mL different VRE bacteria and *E. faecalis* ATCC 29212, that were tested to determine the VRE antimicrobial spectrum as indicator. For inhibition area observation the overlaid plates were incubated overnight and a clear inhibition area of $\geq$3 mm in diameter was reported as positive.

Antimicrobial agents production assay from fermentation broth by cell-free supernatant

Colonies showing inhibition activity were subcultured into 10 mL of TSBYE medium and incubated at 30°C for 24 hours. Then, the broth cultures were inoculated in a 2 L flask containing 500 mL TSBYE without glucose and incubated at 30°C for 24 hours in a rotary shaker with agitation at 180 rpm. Supernatant in the fermentation broth was separated by centrifugation at 13,000 *g* for 10 minutes at 12°C and adjusted to pH 7 with 5 M NaOH. Supernatant was exposed to 100°C for 5 minutes and then filtered using a 0.45 μm-pore-size filter. Some of this supernatant (20 μL) was spotted in BHI agar seeded by *E. faecalis* ATCC 29212 as the indicator. For control, TSBYE medium without glucose and bacteria adjusted to pH 7 with 5 M NaOH and exposed to 100°C for 5 min was used.

Effect of BLIS on the hospital-isolated bacteria

The antimicrobial effect of BLIS of the best producer was investigated on most common hospital-isolated pathogen bacteria, using the spot-on-lawn method as mentioned above. These isolates were identified using standard methods on the basis of their morphological, culture, and biochemical characteristics.

Growth kinetics and BLIS biosynthesis

The maximum BLIS production time in the *Enterococcus* strain that showed the best inhibitory effect on growth of *E. faecalis* ATCC 29212 as the indicator strain, was estimated using bacterial growth curves, based on a method described by Mirhosseini et al.[6] The antibacterial activity was calculated using a critical-dilution micromethod, by assaying serial two-fold dilutions of cell-free culture supernatants against *E. faecalis* ATCC 29212. As described above, cell-free culture supernatants were prepared. Two-fold serial dilutions of the filtrate were made in 100 μL volumes of TSAEY in a 96-well microtest. Each well was then incubated with 40 μL of diluted 100-fold *E. faecalis* ATCC 29212 overnight culture (final concentration of approximately 10^6 CFU/mL). Plates were incubated at 30°C for 18 hours and absorbencies at 630 nm were then measured using ELISA reader (Bio-Tek Microplate Reader, Model EL312, Winooski, United States). This method is based on BLIS activity, expressed in arbitrary units/mL (AU/mL) that is defined as the highest BLIS dilution showing complete inhibition of the indicator strain, and was calculated as the method described by Tahiria et al.[9]

Effects of pH, heat, degenerative enzymes, and exposure to UV light on BLIS

Effects of pH, heat, degenerative enzymes, and exposure to UV light on BLIS activity were tested. To monitor the changes in antimicrobial activity after these treatments, the qualitative spot-on-lawn bioassay, as described above, was used with *E. faecalis* ATCC 29212 as the indicator. For investigation of BLIS stability to temperature, pH and various degenerative enzymes containing proteinase K (pH 7.0), trypsin (pH 7.0) and pepsin (pH 3.0), the method explained by Mirhosseini et al[6] was used. The effect of UV light on BLIS activity was determined as follows: 10 mL of filter-sterilized cell-free supernatant, in a sterile Petri dish, was exposed to UV irradiation at a distance of 25 cm for 5, 10, 15, 20, 25, and 30 minutes. After each time interval, BLIS activity was analyzed by the critical-dilution micromethod as mentioned above.

BLIS molecular weight estimation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and partial purification

For BLIS isolation and partial purification, the best BLIS producer was cultivated in TSBYE for 24 hours at 30°C and then purified partially as the method described by Mirhosseini et al.[6] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for estimation of BLIS molecular weight. After electrophoresis, the gel was fixated by treating with 20% isopropanol and 10% acetic acid for 2 hours. Then, the gel was removed and subdivided into two parts; one part was tested for antimicrobial activity

and the second part was stained with Coomassie Brilliant Blue, containing the protein standards (low-molecular-mass range Sigma Marker protein standards).

Identification of BLSI producing isolate

The identification of the best BLSI producing isolate was initially carried out on the basis of its morphological, culture, and biochemical characteristics using standard methods; Bailey and Scott's Diagnostic Microbiology,[8] and then confirmed using 16S rRNA PCR using DNA extracts with the universal primers RW01 (5′-AAC TGG AGG AAG GTG GGG AT-3′) and DG74 (5′-AGG AGG TGA TCC AAC CGC A-3′) by the CinnaGene Company (Tehran, Iran).

Results

Identification of isolates

All of the 273 *Enterococcus* isolates were obtained from different clinical samples during 1 year. These isolates were Gram-positive, coccus-shaped, aerobes, non spore-formers, tested negative for the catalase enzyme, tested positive for the presence of PYR and had growth ability on bile-aesculin agar and 6.5% NaCl media.

Determination of acquired resistance profiles of *Enterococcus* spp.

To define the acquired resistance profiles in *Enterococcus* isolates, antimicrobial categories and agents were used as in Table 1. Among 273 *Enterococcus* isolates, 212 (77.7%) were MDR, and were sensitive to meropenem, vancomycin, ampicillin, daptomycin, linezolid, quinupristin-dalfopristin and tigecycline but were resistant to other agents. In addition, among 273 *Enterococcus* isolates, 29 isolates were VRE, of which 27 were XDR and two were PDR.

BLIS production assay

Results showed that among the 29 VRE strains, eight isolates had antimicrobial activity showing large inhibition zones against *E. faecalis* ATCC 29212 as the indicator strain. One of these isolates identified as *E. faecium* strain DSH20 based on its phenotypical and biochemical properties, as well as its 16S rRNA gene sequence, had potent BLIS production against all other 29 VRE strains, in addition to the indicator strain. The BLIS production was confirmed in the neutralized supernatant of this isolate (data not shown).

Effect of BLIS on the hospital-isolated bacteria

Different hospital-isolated bacteria containing *Pseudomonas aeruginosa* (*P. aeruginosa*) (10 strains), *Klebsiella* spp. (15 strains), *Acinetobacter* spp. (12 strains), methicillin-resistant *Staphylococcus aureus* (MRSA) (12 strains) and *Escherichia coli* (*E. coli*) (20 strains) were isolated from different patients' samples in the microbiology section of Alzahra Hospital (Isfahan, Iran). These pathogenic bacteria were identified as the essential identification tests on the basis of their morphological, culture, and biochemical characteristics (data not shown). Results shown in Table 2 demonstrated that BLIS of *E. faecium* strain DSH20 had no activity against Gram-negative bacteria containing *P. aeruginosa*, *Klebsiella*, *Acinetobacter,* and *E. coli*. In addition, no activity against MRSA strains was seen.

Table 2 Antimicrobial effect of bacteriocin-like inhibitory substance (BLIS) of *Enterococcus faecium* strain DSH20 on hospital-isolated bacteria

Pathogenic bacteria (*n*)	Size of haloes (mm)
Pseudomonas aeruginosa (10)	0
Klebsiella (15)	0
Acinetobacter (12)	0
Escherichia coli (20)	0
MRSA (12)	0
VRE strains (29)	3—5

MRSA = methicillin-resistant *Staphylococcus aureus*; VRE = vancomycin-resistant *Enterococcus*. A clear inhibition area of $\geq$3 mm in diameter was evidenced as positive.

Growth kinetics and maximum bacteriocin biosynthesis

The growth curves and the profiles of BLIS production of the *E. faecium* strain DSH20 are shown in Fig. 1. BLIS production was started in the mid-exponential phase of growth, and the maximum level (3000 AU/mL) was found at the late-stationary phase of growth. This strain exhibited the maximum antimicrobial activity against *E. faecalis* ATCC 29212 as the indicator strain after 31 hours of incubation at 30°C.

Sensitivity of BLIS to pH, heat, degenerative enzymes, and exposure to UV light

Antimicrobial activity of *Enterococcus faecium* strain DSH20 was stable under heat and acidic/basic treatment, but was inactivated by proteinase K, pepsin, and trypsin enzymes

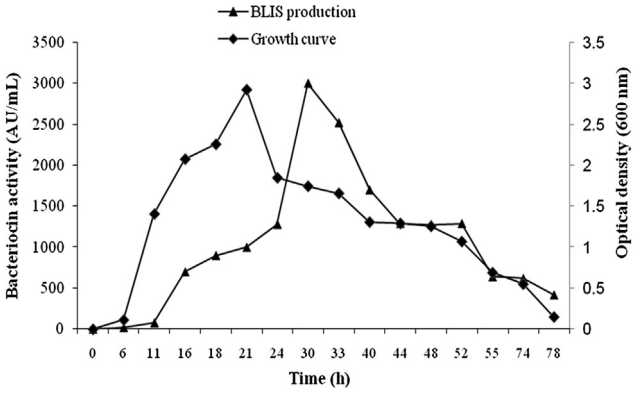

Figure 1. Growth curve and bacteriocin-like inhibitory substance (BLIS) production of *Enterococcus faecium* strain DSH20 determined at 30°C.

Table 3 Effect of different treatments on activity of bacteriocin-like inhibitory substance (BLIS) isolated from *Enterococcus faecium* strain DSH20

Treatment	Activity (%)
Control	100
Degenerative enzymes	
Proteinase K, pepsin, and trypsin	0
Heat	
60°C for 30 min	100
100°C for 10 and 20 min	100
100°C for 45 min	90
121°C for 15 min (autoclave conditions)	75
pH	
3	90
5	90
6—9	100
10	60
UV	
10, 20, and 30 min	100

confirmed by the results of the spot-on-lawn bioassay, showing their proteinaceous nature. The BLIS was active after 30 minutes of exposure to UV light. Results are shown in Table 3.

Estimation of BLIS molecular weight

Partially purified BLIS molecular weight was evaluated by SDS-PAGE, as shown in Fig. 2. The gel stained with Coomassie Brilliant Blue showed protein bands and the molecular weight of partially purified BLIS from *E. faecium* strain DSH20 was estimated to be approximately 35 kDa (Fig. 2). In addition, the SDS-PAGE of BLIS, overlaid with *E. faecalis* ATCC 29212 as the sensitive indicator organism, showed a clear inhibition zone after 24 hours at 30°C (Fig. 3).

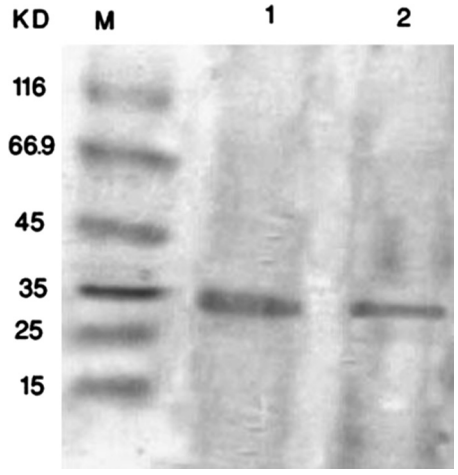

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of partially purified bacteriocin-like inhibitory substance (BLIS) from *Enterococcus faecium* strain DSH20 after staining with Coomassie Brilliant Blue showed the molecular weight of approximately 35 kDa. Line 1 shows a molecular mass marker (in kDa) and lines 2 and 3 show a molecular mass marker of purified BLIS with two repeats.

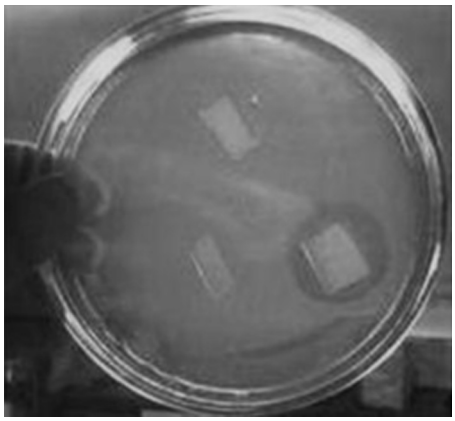

Figure 3. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of bacteriocin-like inhibitory substance (BLIS) overlaying with *Enterococcus faecalis* ATCC 29212 as the sensitive indicator organism showed a clear inhibition zone after 24 hours at 30°C.

Discussion

Enterococci are prominent bacteriocin producers due to production of diversity bacteriocins (enterocins).[10—12] They produce a wide group of structurally diverse antimicrobial peptides, some of which are atypical and different from known bacteriocins.[10] Many bacteriocins from *Enterococcus* have been purified and genetically characterized; most of them have been obtained from *E. faecalis* or *E. faecium*.[10—16] For example, Izquierdo et al,[10] showed that *E. faecium* IT62 produces three different bacteriocins, EntL50A, EntL50B, and EntIT. Birri et al,[12] isolated bacteriocin-producing *E. avium* isolates, and a new pediocin-like bacteriocin (termed avicin A) was purified and characterized. Nilsen et al,[15] isolated a novel antimicrobial protein, designated enterolysin A from an *E. faecalis* LMG2333 culture that inhibits growth of selected *enterococci*, *pediococci*, *lactococci*, and *lactobacilli*. Kang and Lee,[13] isolated *E. faecium* GM-1 produces a new bacteriocin that has a broad antibacterial spectrum including both Gram-positive and Gram-negative bacteria. Nascimento et al,[14] investigated the antimicrobial activity of *E. faecium* FAIR-E 198 against *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus*. Zendo et al,[17] reported bacteriocin production by *E. mundtii* QU2 that inhibited the growth of various indicator strains, including *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Listeria*.

Despite these wide studies about bacteriocins production in *Enterococcus* isolates, there are rare studies about bacteriocins production in *Enterococcus* that have broad spectrum activity against PDR strains of VRE; there are no antibiotics for the treatment of these strains. Recent emergence of these pathogen bacteria has become a major clinical problem mobilizing the search for new potent antimicrobial agents.[1,2] Aunpad and Na-Bangchang[11]

isolated pumilicin 4, a novel bacteriocin from *Bacillus pumilus* strain WAPB4 with anti-MRSA and anti-VRE activity, and Yamashita et al,[16] isolated bacteriocin 51 (Bac 51) from vancomycin-resistant *E. faecium* VRE38 that was active against *E. faecium*, *E. hirae*, and *E. durans*. In this study, *Enterococci* were isolated from different clinical samples and screened for broad spectrum bacteriocin production. One *Enterococcus* isolate identified as *E. faecium* strain DSH20 had potent BLIS production against all tested VRE strains, in addition to the indicator strain.

BLIS produced by *E. faecium* strain DSH20 inactivated by proteinase K, pepsin, and trypsin enzymes, provided evidence for its proteinaceous nature, showed stability at a wide range of pH values from pH 3.0—9.0 at 30°C, was heat stable even after heating at 100°C for 5 minutes and kept at 75% in autoclave conditions (121°C for 15 minutes). In addition, the BLIS retained its activity after 30 minutes of exposure to UV light, which is the first report for this isolate. During the stationary phase of growth, the maximum levels of antimicrobial activity were determined according to findings by others.[1,6]

The molecular weight of this BLIS was approximately 35 kDa. Some other bacteriocins produced by strains from the *Enterococcus* genus, like pumilicin 4 isolated by Aunpad and Na-Bangchang,[11] as determined by mass spectrometry, was 1994.62 Dalton, have molecular weights ranging between 7 and 20 kDa and therefore most of these reported bacteriocins have molecular masses lower than that of our BLIS. This BLIS seems to be is a novel BLIS with a high molecular weight and it needs further identifying using a method such as MALDI-TOF; the potential gene encoding this BLIS needs to be provided.

Ideal physicochemical properties of this BLIS and its broad range of activity against VRE isolates, strongly suggests its use in preserving a wide range of foods or in pharmaceutical compositions that require heating during preparation, and as an alternative antibacterial agent for treatment of drug-resistant strains of VRE infection.

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