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STUDIES ON BREVICIN AF01: A BACTERIOCIN LIKE INHIBITORY SUBSTANCE ACTIVE AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

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Abstract

Bacillus brevis AF01, isolated from wheat field, has been found to produce proteinaceous antibacterial substance Brevicin AF01. The inhibitory activity could not be related to bacteriophages. It has a narrow spectrum of activity against gram-positive bacterial strains only. Brevicin AF01 revealed bactericidal effect against *Staphylococccus aureus* FS10. It was also found to be active against several strains of methicillin resistant *Staphylococcus aureus.* Its production was started in the early stationary phase and continued till late stationary phase. Maximum production of Brevicin AF01 was observed when the producer strain was grown in BHI broth without NaCl supplement, at neutral pH and at 37°C. It remained stable at 60°C and 80°C for 30 minutes. The activity decreased at 100°C and was completely lost by autoclaving (121°C, 15 lb/sq inch, for 15 minutes). It remained stable at wide pH range (3-11). Bioactivity of Brevicin AF01 was relatively less affected by treatment with organic solvents, heavy metal salts but lost completely by surfactants and proteolytic enzymes. The activity was also found sensitive to lipase and thus Brevicin AF01 appeared to be a lipoprotein.

Introduction

Staphylococcus aureus cause a number of varied infections ranging from skin abscesses to bone and soft tissue intra-surgical infections, sepsis and invasive endocarditis (Lowy, 1998; Chambers, 2001). Due to institutional overuse of antibiotics, strains of *Staphylococcus aureus* have developed drug resistance. Methicillin-resistant *Staphylococcus aureus* (MRSA) constitute specific bacterial strains that have developed antibiotic resistance to all penicillins, including methicillin and other narrow-spectrum β-lactamase-resistant penicillin antibiotics (Foster, 1996). Resistance to methicillin is encoded by the *mec*A gene and expression of this gene yields PBP2a, a penicillin binding protein with reduced affinity for β-lactam rings, the primary active-site of the β-lactam antibiotics (Guignard *et al*., 2005). Classically, MRSA has been a nosocomial problem but now it appeared to be a community pathogen. MRSA infections pose a serious threat to patient care. Although antibiotics are available for the treatment of MRSA infections, because of numerous adverse effects and resistance to these antibiotics there is an urgent need to search for alternatives to synthetic antibiotics for treating MRSA infections.

Antimicrobial peptides are secreted by both gram-positive and gram-negative bacteria and among them bacteriocins/bacteriocin like inhibitory substances are the most important. Bacteriocins are ribosomally synthesized antimicrobial peptides with bactericidal activity towards species that are often closely related to the producer bacteria (Tagg *et al*., 1976). The producer cell exhibits genetically encoded immunity to the action of its own bacteriocin (Nes *et al.*, 1996). They are heterogeneous compounds that display

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variable molecular weights, biochemical properties and inhibitory spectra (Sullivan *et al*., 2002). The mechanisms of action of bacteriocins are diverse, but the bacterial membrane is the target for most bacteriocins (Klaenhammer, 1993). Several uncharacterized substances with bacteriocin-like activity have been identified and are referred to as bacteriocin-like inhibitory substances (BLIS/BLS) (Daw & Falkiner, 1996). Since bacteriocins are natural substances, there is tremendous interest in their use as biopreservatives as well as therapeutic agents (Cleveland *et al*., 2001).

Several bacteriocins or bacteriocin-like substances (BLS) produced by the genus *Bacillus* have been reported eg., a bacteriocin of *B. brevis* (Hyung *et al*., 2001), subtilin by *B. subtilis* (Klein *et al*., 1992), bacteriocin of *B. cereus* (Oscariz & Pisabarro, 2000), thruicin 7 by *B. thuringiensis* (Cherif *et al*., 2001) and many others. The primary role of these antimicrobial agents is to provide an advantage over other competitive organisms occupying the same ecological niche. Both narrow spectrum (Risoen *et al*., 2004) and broad spectrum (Teo & Tan, 2005) bacillocins have been characterized. Several bacillocins have been reported to inhibit *Staphylococcus aureus* and also MRSA strains like mersacidin, a lantibiotic (lanthionine containing bacteriocin) produced by *Bacillus* sp. HIL Y-85, 54728 (Altena *et al*., 2000) can successfully inhibit the growth of MRSA and other gram-positive bacteria *In vitro* (Hoffmann *et al*., 2002). Present studies were carried out to search for alternatives to synthetic antibiotics against MRSA.

Material and Methods

Bacterial strains and culture media: Fifteen methicillin resistant and 35 methicillin sensitive *Staphylococcus aureus* strains were collected from different pathological laboratories of Karachi and were identified on the bases of morphological and cultural characteristics. Methicillin resistance profile was determined by agar disc diffusion assay as described by Cetin & Gurler (1989). *Bacillus brevis* AF01 was obtained from Laboratory of Molecular Genetics (LMG), Department of Microbiology, University of Karachi. Brain Heart Infusion (BHI) agar was used as routine medium for growing producer and indicator/sensitive strains and brain heart infusion broth containing 40% glycerol (Fischer Scientific Co Raleigh, USA) was used to maintain the stock cultures. Brain heart infusion soft/overlay agar was prepared by adding 0.6% granulated agar (Oxoid) to BHI broth. Before experimental use, the cultures were propagated twice in BHI broth.

Assays for bacteriocin activity: The following three methods were used for the detection of bacteriocinogenic potential of the strains used in the study as described by Iqbal *et al*., (1999): 1) Stab-overlay method; 2) Cross-streak method and 3) Agar-well diffusion assay.

Bacteriocin activity manifestation or lytic action by phages: To investigate the possibility that either viable or defective phage particles were present in supernatants of the producer strains and responsible for the inhibitory activity, a disc of agar from 24h old stabbed cultures (diameter of its usual inhibition zone) of producer strain was taken and crushed in tubes containing sterile broth and then activity was checked by agar well diffusion assay (Gagliano & Hinsdill, 1970).

Titration of bacteriocin like inhibitory substance: Bacteriocin activity (titre) was determined in terms of arbitrary units (AU) by a method described by Parente *et al*., (1995).

BLIS production under different conditions: To monitor the production of BLIS under different conditions the producer strain (*Bacillus brevis* AF01) was grown in five different media *viz*., Tryptic Soy broth (TSB), Nutrient broth, Brain Heart Infusion (BHI) broth, Lactose broth and Luria basal broth (LBB)] at different incubation temperatures of 20, 29, 37, 45 and 53ºC. The production was also monitored in BHI broth tubes having different pH values (between 4 and 10) and also in BHI broth tubes containing different concentrations of 0, 0.5, 1, 1.5, 2 and 2.5% NaCl. All tubes were incubated at 37°C for 18-24 hours. Activity was checked by agar well diffusion assay (Ogunbanwo *et al*., 2003).

Physicochemical characterization of BLIS: Physicochemical characterization of BLIS (Brevicin AF01) which was produced by *Bacillus brevis* AF01, was done which included effect of enzymes (proteinase K, trypsin, protease and lipase); effect of temperatures (40, 60, 80 and 100°C for upto 30 minutes and at 121°C, 15 lb/sq. inch for15 minutes); effect of pH (range 1-14); effect of organic solvents (methanol, ethanol, propanol, butanol, and chloroform); effect of heavy metal salts ($AgNO_3$, $MgSO_4$, $MnCl_2$, $ZnSO_4$, $CuSO_4$, $CdCl_2$, $CsCl_2$, $NiSO_4$, $FeSO_4$ and $BaCl_2$) and effect of surfactants [Tween20, Tween80, Sodium dodecyl sulphate (SDS) and Ethylene diamine tetra acetic acid (EDTA)]. Activity was checked by agar well diffusion method (Prasad *et al*., 2005).

Mode of action and growth curve with simultaneous measurement of Brevicin AF01 production: To investigate mode of action of Brevicin AF01, the method described by Bhunia *et al*., (1991) was applied. Synthesis of BLIS by *Bacillus brevis* AF01 was monitored during the growth cycle by using the method of Iqbal *et al*., (1999).

Ammonium sulphate precipitation: Ammonium sulphate precipitation of BLIS was done by the method described by Cherif *et al*., (2001).

Results and Discussion

Production of antagonistic substances is an important factor in microbial ecology. Among the many different substances known to play a role in bacterial interactions, bacteriocins are the most specific yet efficient antagonists. Bacteriocins are the peptide antibacterial substances with bactericidal activity directed against species, which are usually closely related to the producer microorganisms. However, it has been indicated that bacterial protein toxins may also prove to be broad spectrum antagonists (Rasool & Akhter, 1992). Although *Bacillus* spp. appear to be a relatively abundant source of antimicrobials, since many species of this genus synthesize antimicrobial peptides, only limited data exist on bacteriocins from *Bacillus brevis* (Stein, 2005).

Bacterial strains: All bacterial strains were collected from different pathological laboratories of Karachi and identified on the bases of morphological and cultural characteristics (Easmon & Adlam, 1983). Methicillin resistance profile of MRSA strains was determined and results are shown in Table 1.

Table 1. Methicillin resistance profile of test strains of *Staphylococcus aureus*

Methicillin resistant *Staphylococcus aureus* strains	Diameter of zones of inhibition (mm)
Staphylococcus aureus FS1	0
Staphylococcus aureus FS2	0
Staphylococcus aureus FS3	0
Staphylococcus aureus FS4	0
Staphylococcus aureus FS5	0
Staphylococcus aureus FS6	6
Staphylococcus aureus FS7	0
Staphylococcus aureus FS8	0
Staphylococcus aureus FS9	0
Staphylococcus aureus FS10	0
Staphylococcus aureus FS11	7
Staphylococcus aureus FS12	0
Staphylococcus aureus FS13	6
Staphylococcus aureus FS14	0
Staphylococcus aureus FS15	0

Antibiotic sensitivity was checked by disc diffusion assay (excluding the size of antibiotic disc)
Key: 0 = No zone of inhibition.

Table 2. Inhibitory spectrum of Brevicin AF 01

Sensitive/indicator strains	A
Methicillin resistant *Staphylococcus aureus*	9/15
Bacillus subtilis	1/1
Staphylococcus aureus	2/2
Staphylococcus epidermidis	3/3
Streptococcus pyogenes	1/1
Enterococcus fecalis	1/1
Micrococcus luteus	1/1
Corynebacterium diphtheriae	1/1
Corynebacterium xerosis	1/1
Klebsiella pneumoniae	0/1
Pseudomonas aeruginosa	0/1
Escherichia coli	0/1

Activity was checked by cross streak method.
Key: A= Number of sensitive strains/ number of tested strains

Screening for BLIS activity: All the isolates were screened for bacteriocinogenic potential by three different methods i.e., stab-overlay method, cross-streak method and agar-well diffusion assay. *Bacillus brevis* AF01 was found to produce a BLIS, Brevicin AF01 which was inhibitory against nine out of fifteen MRSA strains (Table 2). Bacteriocins from *Bacillus* species are known to exert inhibitory effect on various gram-positive bacteria other than closely related species including *Staphylococcus aureus*. A bacteriocin cerecin 7 from *Bacillus cereus* was found to inhibit the growth of *Staphylococcus aureus* ATCC12600 (Oscariz & Pisabarro, 2000). Reportedly, mersacidin, a lantibiotic produced by *Bacillus* sp. HIL Y-85, 54728 inhibited the growth and colonization of methicillin resistant *Staphylococcus aureus* (Kruszewska *et al*., 2004).

Activity spectra: Bacteriocins of gram-negative bacteria usually have narrow spectrum of activity but those produced by gram-positive bacteria may have a wide range of spectrum of action (Hardy, 1982). The isolate (*Bacillus brevis* AF01) resisted the inhibitory activity of its own product (Hanlin *et al*., 1993). The inhibitory activity of Brevicin AF01 was found narrow spectrum and restricted to only gram-positive bacteria (Table 2). Similar findings were reported by Cherif *et al*., (2001) in which thuricin 7, a bacteriocin from *Bacillus thuringiensis* BMG1.7 was found to be inactive against gram-negative bacteria. In contrast to the present study, bacteriocin produced by *Bacillus brevis* was found to have broad spectrum of bioactivity against both gram-positive and gram-negative bacteria (Hyung *et al*., 2001).

Bacteriocin activity manifestation or lytic action by phages: The possibility that either viable or defective phages were responsible for the antibacterial activity has also been ruled out. Bacteriocins, unlike bacteriophages do not carry the genetic determinants necessary for self replication. Thus, when a block from zone of inhibition was cut and emulsified in sterilized buffer it did not give any zone of inhibition thereby suggesting that the inhibitory molecules constituted bacteriocin/bacteriocin like inhibitory substances and not the bacteriophages.

BLIS production under different conditions: Although, bacteriocin production is thought to be under complex genetic regulation, its production is influenced by several environmental factors, such as pH (Nilsen *et al*., 1998), temperature (De Vuyst *et al*., 1996) and NaCl (Uguen *et al*., 1999). Maximal bacteriocin production could be obtained by supplementing a culture medium with growth limiting factors such as sugars, vitamins and nitrogen sources, by regulating pH or by choosing the best-adapted culture medium (Vignolo *et al*., 1995). Ethanol concentrations also affect the production of bacteriocin (Abildgaard *et al*., 1995). Maximum production of Brevicin AF01 was observed when *Bacillus brevis* AF01 was grown in BHI broth (without NaCl supplement and at neutral pH) at 37°C (Figs. 1 and 2). A similar type of observation has been reported by Lisboa *et al*., (2006) who have studied the effect of media on the production of BLIS by *Bacillus amyloliquefaciens*.

Physiochemical characterization of BLIS: The antimicrobial substance (Brevicin AF01) was completely inactivated by treatment with trypsin and a decrease in the activity was observed when it was treated with protease and proteinase K, suggesting that a peptide moiety is associated with its bioactivity. Activity of BLIS was also found sensitive to lipase which indicated that lipid component was involved in antibacterial activity as well (Table 3). A bacteriocin with active lipid moiety (produced by *Bacillus cereus*) has also been reported by Torkar & Matijasi (2003). Brevicin AF01 was relatively thermostable (Table 3). The pattern of heat resistance by Brevicin AF01 resembles with a BLIS produced by *Bacillus licheniformis* P40 (Olivera *et al*., 2004).

The activity of Brevicin AF01 was pH dependant. It was found to be active over a wide range of pH (3-11). The results are in agreement with the findings of Hyung *et al*., (2001). Brevicin AF01 retained about 80-90% of the bioactivity after treatment with organic solvents. These results are in consonance with the ones observed by Hyung *et al*., (2001). Brevicin AF01 retained about 80-93% of the activity after treatment with most of the heavy metal salts used in the study, however only 65-73% of bioactivity was detected when Brevicin AF01 was treated with Cd Cl_2 and $CuSO_4$. Exposure of the BLIS preparation to surfactants like Tween20, Tween 80 and SDS resulted in the loss of antibacterial activity (Table 3). Inhibition of the bioactivity of Brevicin AF01 by SDS is also indicative of its proteinaceous nature as suggested by Prasad *et al*., (2005).

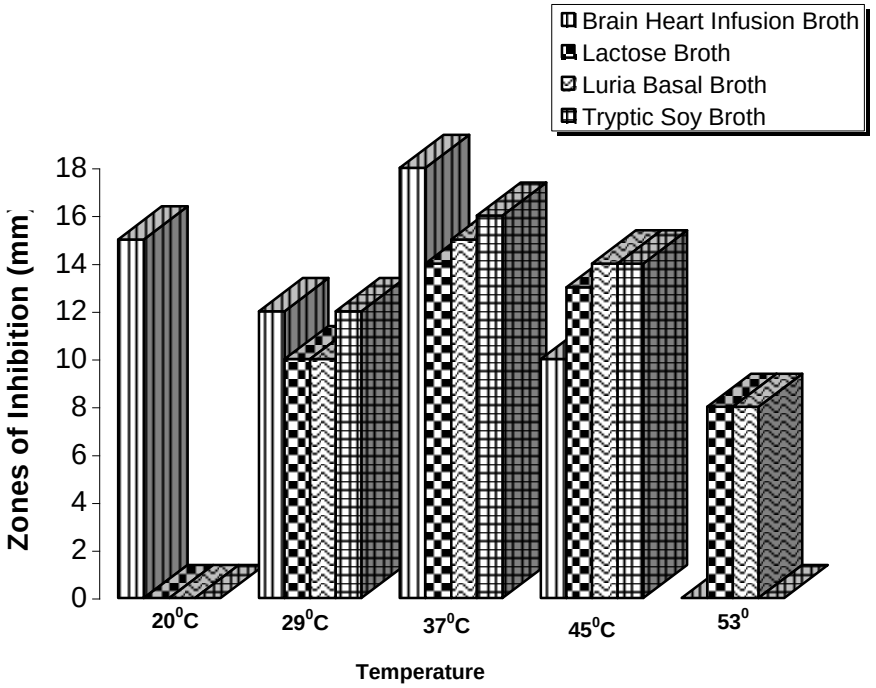

Fig. 1. Effect of different media on the production of Brevicin AF01 at different incubation temperatures.

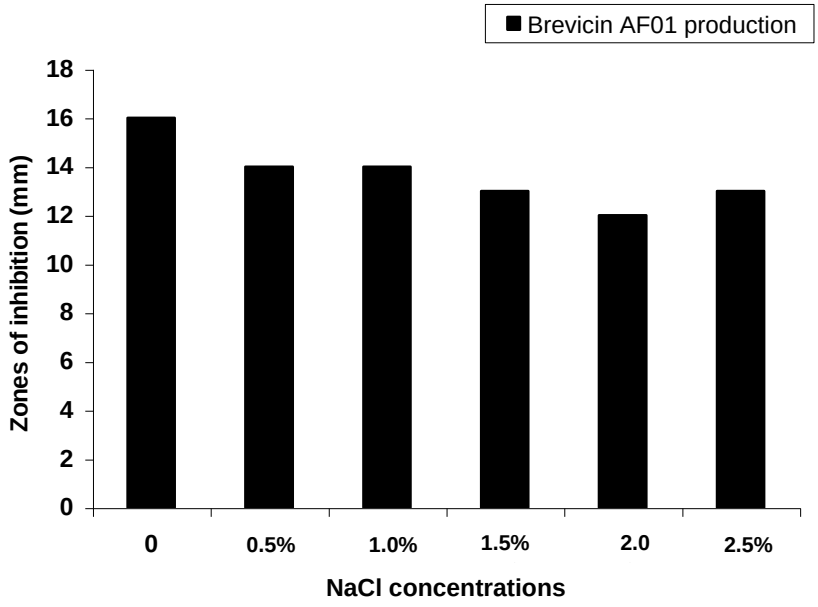

Fig. 2. Effect of NaCl on Brevicin AF01 production.

Table 3. Physicochemical characterization of BLIS.

	Treatments	Bioactivity
1. Enzymes:	Protease	+
	Proteinase K	+
	Lipase	-
	Trypsin	-
2. Temperature:	40°C (30 min)	+
	60°C (30 min)	+
	80°C (30 min)	+
	100°C (30 min)	+
	121°C (15 lb/sq inch, for 15 min)	-
3. pH:	1	-
	3	+
	5	+
	7	+
	9	+
	11	+
	14	-
4. Organic solvents		+
5. Heavy metal salts		+
6. Surfactants (except EDTA)		-
7. EDTA		+
Arbitrary Units/mL		320AU/mL

Key: + =activity retained, - = loss of activity.

Mode of action: *Bacillus* species produce many bacteriocins/bacteriocin like inhibitory substances, which show bactericidal (Bizani *et al*., 2005) or bacteriolytic activity (Cherif *et al*., 2001). The effect of Brevicin AF01 on the log phase and stationary phase cells of the indicator strain was monitored. Log phase as well as stationary phase cells showed a decrease in the cell viability after addition of Brevicin AF01 suggesting a bactericidal mode of action (Figs. 3 and 4).

Ammonium sulphate precipitation: Ammonium sulfate precipitation is a procedure used for the concentration of proteins. Brevicin AF01 was successfully precipitated at 80% ammonium sulfate saturation since a marked increase in the activity was observed. Thuricin 7, a bacteriocin from *B. thuringiensis* was also precipitated at 80% ammonium sulfate saturation (Cherif *et al*., 2001).

The above discussion based findings are clearly reflective of the fact that MRSA have been found sensitive to the novel bacteriocin like substance Brevicin AF01 and after further investigative approaches, one may find solution to contain and counter the MRSA associated infections (both the Community acquired and Hospital acquired ones).

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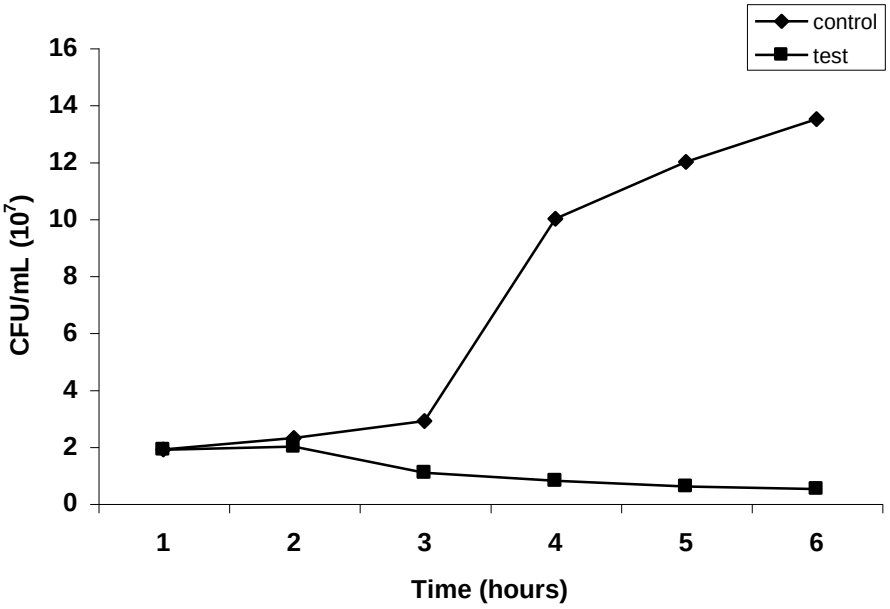

Fig. 3. Mode of action of Brevicin AF01 on Stationary phase cells of *Staphylococcus aureus* FS10.

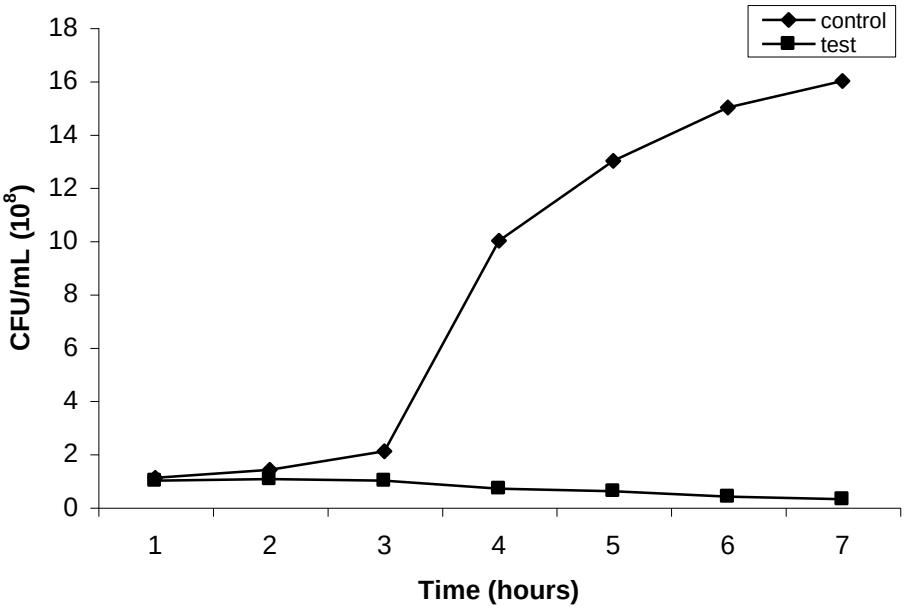

Fig. 4. Mode of action of Brevicin AF01 on actively growing (log phase) cells of *Staphylococcus aureus* FS10.

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