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ANTIMICROBIAL SPECTRUM, PRODUCTION AND MODE OF ACTION OF STAPHYLOCOCCIN 188 PRODUCED BY *STAPHYLOCOCCUS AUREUS* 188

SADIA SAEED, SAMIA AHMAD AND SHEIKH AJAZ RASOOL
Department of Microbiology, University of Karachi, Karachi-75270

ABSTRACT

Staphylococcus aureus 188 has been shown to produce bacteriocin-like inhibitory substance known as staphylococcin188. It has a broad-activity spectrum against *Micrococcus luteus*, *Streptococcus pneumoniae*, *Streptococcus faecalis*, *Streptococcus viridans*, *Corynebacterium diphtheriae* and several staphylococcus species. The arbitrary unit of staphylococcin 188 against *Micrococcus luteus* was 1280AU/mL. Its production with simultaneous measurement of activity was monitored and was found to produce maximum amount of staphylococcin after 7 hours of incubation. Mode of action of the staphylococcin 188 on the sensitive cells was bactericidal rather than bacterioloytic.

INTRODUCTION

Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria (Cleveland *et al*., 2001). The term bacteriocin-like inhibitory substances (BLIS) is applied to antagonistic substances which are not completely defined or do not fit the typical criteria of bacteriocins. They have been reported to inhibit a wide range of both Gram-positive and Gram-negative bacteria. In recent years, interest have been shown on the microbiology, biochemistry & molecular biology of bacteriocin like inhibitory substances (BLIS) because they are medically, industrially & agriculturally very important (Riley and Wertz, 2002a, 2002b). Bacteriocins have been effective in controlling bacterial infections and their extensive use in combinations as natural food bio-preservatives and health care products has attracted many researchers (Hanlin *et al*., 1993; Schillinger and Lucke, 1989). Staphylococcal bacteriocins have been the subject of much study over the past few decades and several reports describe their production, purification and characterization (Hale and Hinsdil 1973, Navartana *et al*., 1998; Iqbal *et al*., 1999, 2001). Bacteriocins produced by staphylococci are active not only against other staphylococci but also against some other microorganisms (De-Oliveira *et al*., 1998).

Understanding the significance particularly with reference to the therapeutic potential of staphylococcin, we have decided to determine the spectrum, production and mode of action staphylococcin 188 in order to explore the applied aspects and a possibility of this bacteriocin for its extra laboratory application.

MATERIALS AND METHODS

Activity spectrum of staphylococcin 188

Following two methods were used to determine the activity spectrum of staphylococcin 188. All the experiments performed in this study were modified according to our laboratory conditions and carried out in triplicates.

1) Stab & overlay method:

Brain heart infusion agar plate was stabbed with the *S. aureus* 188 and incubated at 37°C for 24h. Next day plate was exposed to chloroform vapours to kill the producing strain (Keeping the plates inverted and 9cm piece of Whatman No.1 filter paper was introduced into the lid and impregnated with 1mL of chloroform for 20-30 min). Plate was then overlaid with 3mL soft agar containing 0.1mL of indicator/sensitive organism. Plate was again incubated at 37°C for overnight and observed for clear zone around the producer culture (Schillinger *et al*., 1991).

2) Agar- well diffusion assay:

Brain Heart Infusion agar plate was overlaid with 3mL BHI soft agar containing 0.1mL of the indicator/sensitive culture. Wells were cut into agar plates and 100µl of staphylococcin 188 was placed into each well. The plates were incubated and zones of inhibition were measured in mm (Cooper and James, 1984). The bacteriocin activity was expressed as arbitrary units/mL. An arbitrary unit (AU/mL) is defined as 100µL of the highest dilution of the preparation yielding a definite zone of inhibition on the lawn of the sensitive cells of *M. luteus* (Rasool *et al.,* 1996).

Growth cure with simultaneous measurement of staphylococcin 188 production:

Synthesis of staphylococcin188 was monitored during the growth cycle by growing the producer culture *S. aureus* 188 overnight in 5mL BHI broth. Next day optical density at 600nm (spectrophotometer, spectronic-21, Bausch and Lomb) was measured and 1mL of the culture was transferred to 150mL of fresh broth and incubated at 37ºC. After every hour optical density was recorded, 0.1mL samples were plated on BHI agar medium to score the colony forming unit (cfu/mL) and 1.0mL samples were centrifuged (10,000rpm, for 30min) and supernatants were assayed for bacteriocin activity by the agar-well diffusion method as described by (Iqbal *et al*., 1999).

Mode of action of staphylococcin 188 on indicator/sensitive cells

a) Effect of staphylococcin 188 on stationary phase cells of *M. luteus*:

M. luteus was grown overnight in BHI broth at 37°C harvested by centrifugation (10,000xg for 10 min) and re-suspended in 50mM sodium phosphate buffer (pH.7). Two-fold serial dilution of staphylococcin 188 was made in the same buffer and 0.2mL of *M. luteus* cells was added to each dilution (1.8mL) of staphylococcin 188. Control was 0.2mL culture without adding staphylococcin 188, instead making up the volume with 1.8mL buffer. The mixture was incubated, and the samples were drawn at 0, 0.5, 1, 1.5, 2.0 and 4.0 hours. Absorbance of each sample was read at 600nm (Bhunia *et al*., 1991) .

b) Effect of staphylococcin on growing cells of *M. luteus*:

Actively growing cells *M. luteus* was diluted 100 times in tempered BHI broth and incubated at 37°C. After 2h of incubation, 1mL (of each two-fold dilution) of staphylococcin 188 was added to 10mL of the *M. luteus* culture and incubated at 37°C. The mixtures were incubated, and the samples were drawn at 0, 0.5, 1.0, 2.0 and 4.0 hours. Absorbance of each sample was read at 600nm.

RESULTS

Staphylococcus aureus 188 was tested for its bacteriocinogenic potential by two methods i.e. stab & overlay and agar-well diffusion method. Reason of using two methods was to compare some of the most commonly used methods for the detection of bacteriocin activity. In our studies it was found to produce bacteriocin/staphylococcin by both the methods. A number of bacterial

genera belonging to both inter and intra-generic Gram-positive group and some fungi were tested as sensitive/indicator cultures. Interestingly, it was found active against *Staphylococcus aureus, Staphylococcus epidermidis, Staphlococcus saprophyticus, Streptococcus viridans, Streptococcus pnenomoniae, Streptococcus faecalis, Sarcinae species, Micrococcus luteus* and *Corynebacterium diphtheriae* but not against *Candida albicans* and *Saccharomyces cerevisiae* (Table I). Figure 1 shows the stab & overlay method and figure 2, agar-well method to demonstrate staphylococcin 188 activities against *M. luteus*. In stab & overlay method chloroform vapours were used to kill producer culture, there was no change in size of zone of inhibition was observed, suggesting that chloroform has no effect on staphylococcin activity. Bacteriocin titer was expressed as activity

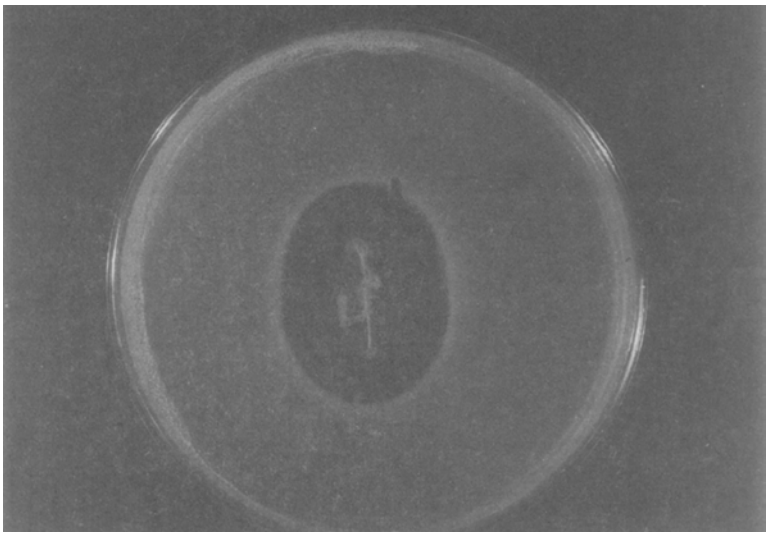


Fig. 1: *Staphylococcus aureus* 188 showing staphylococcin activity by stab & overlay method against *Micrococcus luteus* as sensitive culture.

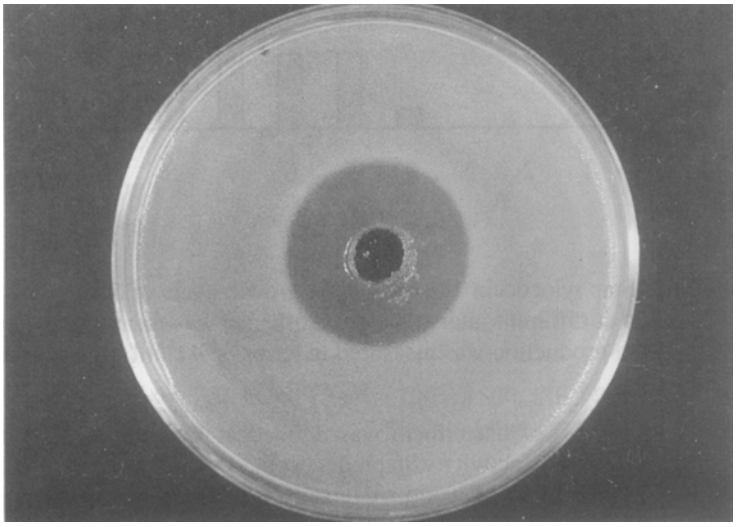


Fig. 2: *Staphylococcus aureus* 188 showing staphylococcin activity by agar-well diffusion method against *Micrococcus luteus* as sensitive culture.

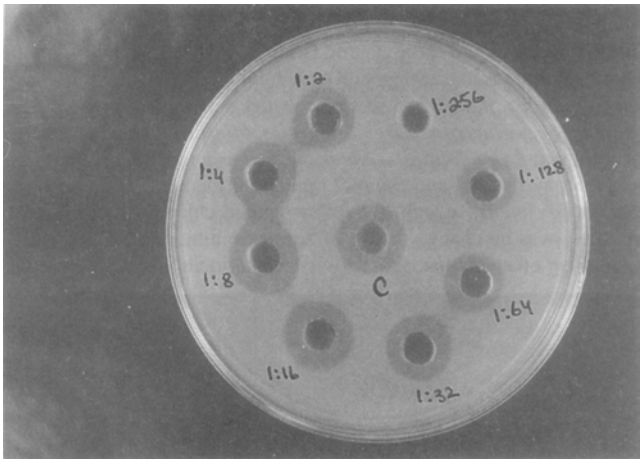
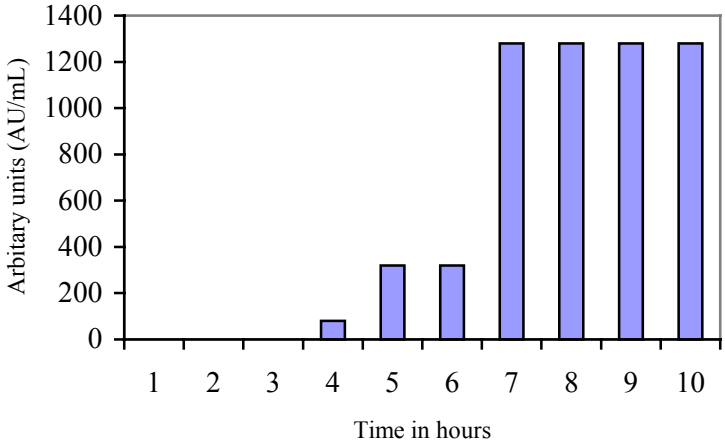


Fig.3: Agar-well diffusion method demonstrating staphylococcin 188 activity in terms of activity units per mL (AU/mL) against *Micrococcus lureus*.

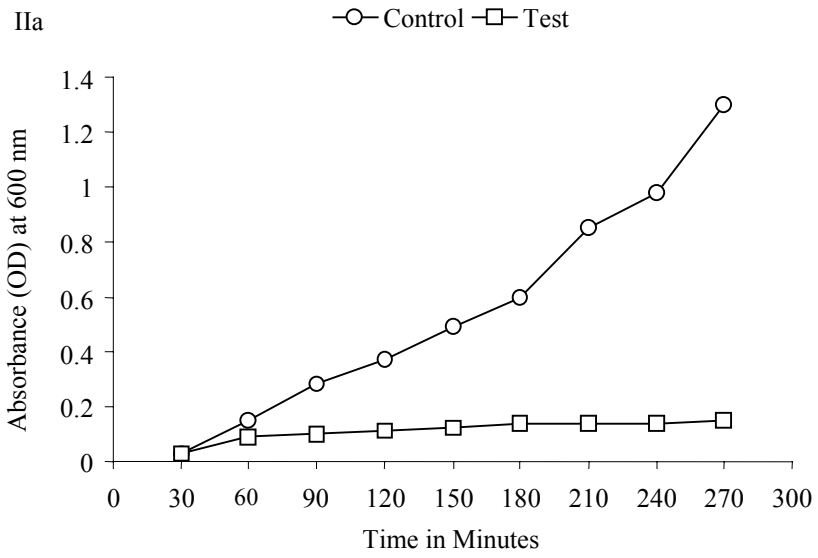
$$\text{AU/mL} = \frac{\text{Reciprocal of the highest dilution x 1000}}{\text{Volume of staphylococcin}}$$

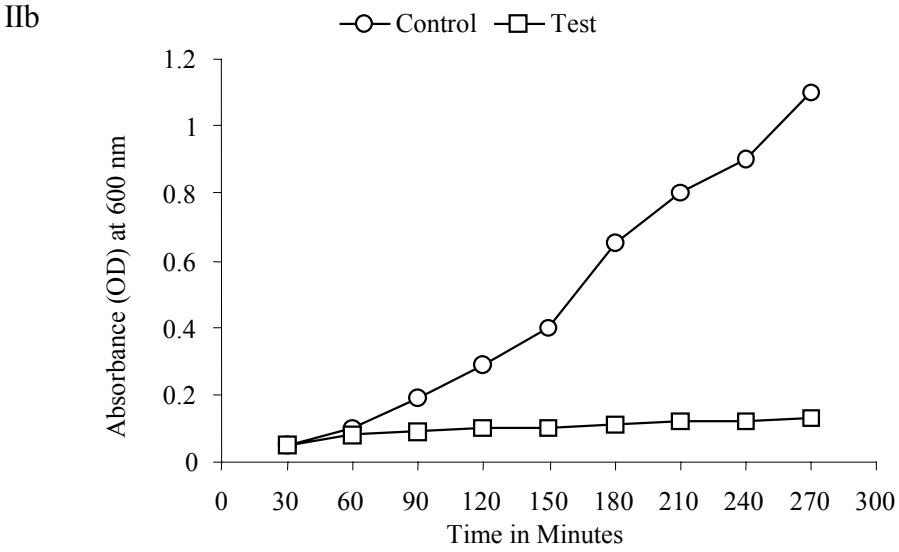


Graph 1: Production of Staphylococcin 188 during the growth cycle of *Staphylococcus aureus* 188. Samples were taken at different intervals and absorbance was measured in terms of O.D. at 600nm. Staphylococcin production was measured in terms of AU/mL by agar-well diffusion method.

unit/mL. One activity unit (AU) of bacteriocin was defined as the reciprocal of the last serial dilution demonstrating inhibitory activity. Staphylococcin 188 titer against *M. luteus* was estimated to be 1280AU/mL (figure 3). The growth curve of *S. aureus* 188 was run to find out the critical phase of growth cycle offering maximum staphylococcin production (graph 1). Staphylococcin 188 production starts during early logarithmic phase, as the culture supernatant of *S. aureus* 188 was found to contain staphylococcin after 4th hours of incubation and activity

reaches to maximum at 7th hour and then remained stabled throughout incubation period. To investigate the antagonistic effect of bacteriocin was whether bactericidal or bacteriolytic, the effect of staphylococcin 188 on logarithmic and stationary phase cells of *M. luteus* was examined. The results depicted in graph 2a and 2b indicates its bactericidal effect, as the optical density of the cell suspension remains constant throughout the course of incubation.





Graph IIa & IIb: Bactericidal effect of staphylococcin 188 on (IIa) logarithmic and (IIb) stationary phase cells of *Micrococcus luteus*. Samples were obtained at different time intervals and absorbance was measured in terms of OD at 600 nm.

Table
Inhibitory spectrum of staphylococcin 188 against inter and intra-generic Gram-positive bacteria and Fungi

Sensitive/indicator organism	**Zone of inhibition (mm)**
Gram-positive bacteria (intra-generic)	
Micrococcus luteus	**35**
Staphylococcus aureus	**23**
Staphylococcus epidermidis	**19**
Staphylococcus saprophyticus	**20**
Gram-positive bacteria (inter-generic)	
Bacillus cereus	**-**
Bacillus subtilis	**-**
Corynebacterium diphtheriae	**27**
Streptococcus pyogenes	**15**
Streptococcus pneumoniae	**25**
Streptococcus faecalis	**29**
Streptococcus viridans	**24**
Sarcina spp.	**35**
Fungi	
Candida albicans	**-**
Saccharomyces cerevisiae	**-**

DISCUSSION

Bacteriocins, antimicrobial peptides, and bacteriophage have attracted attention as potential substitutes for, or as additions to, currently used antimicrobial compounds. They are proteinaceous compounds of bacterial origin that are lethal to bacteria other than the producing strain. It is assumed that some of the bacteria in the intestinal tract produce bacteriocins as a means to achieve a competitive advantage, and bacteriocin-producing bacteria might be a desirable part of competitive exclusion preparations. Purified or partially purified bacteriocins could be used as preservatives or for the reduction or elimination of certain pathogens. Currently only nisin, produced by certain strains of *Lactococcus lactis* subsp. lactis, has regulatory approval for use in certain foods, and its use for poultry products has been studied extensively (Navarro *et al.,* 2000 and Joerger, 2003).

The present research work associated with activity spectrum, production and mode of action of staphylococcin 188 produced by *Staphylococcus aureus* 188. In our studies the inhibitory spectrum of staphylococcin 188 was exhibited against both inter and intra-generic Gram-positive bacteria and the producer strain was resistant to its own bacteriocin. Staphylococcin 188 besides inhibiting closely related staphylococcal species including *S. aureus, S. epidermidis and S. saprophyticus*, also inhibit other inter-generic bacterial strains such as *S. viridans, S. faecalis, S.*

pneumoniae, *M. luteus, C. diphtheriae* and *Sarcinae* species. These results indicate that staphylococcin 188 has a broad spectrum of activity. Earlier, (Crupper *et al.,* 1997) also reported broad activity spectrum of staphylococcin Bac R1. Chloroform was used in stab & overly method to kill the producer culture and subsequently overlaying with indicator culture (Barrow, 1963). Rasool *et al.,* (1996) had reported that two out of twelve streptocins were sensitive to chloroform vapours. In our case staphylococcin 188 was resistant and its activity retained even after 20-30min exposure to chloroform vapours. Activity unit of Staphylococcin 188 was found to be 1280/mL in two-fold serial dilution using *M. luteus* as sensitive culture. Maximal bacteriocin yields in a culture may occur at a different phases of the growth cycle. In our studies production of staphylococcin 188 in brain heart infusion broth starts from 4^{th} hour of incubation, its activity reaches to maximum level at 7^{th} hour and then remained stable throughout the incubation period. This prolonged stability of staphylococcin 188 in the growth medium is similar to that of other Gram-positive bacteriocins (Crupper and Landolo, 1996; Schobitz *et al*., 2003). Lachowicz (1965) studied the dynamics of staphylococcin A-126a production on solid media, where activity was first detected after 8 hours of incubation, reached a maximum at 18 to 24 hours and subsequently fell to zero. Staphylococci produce inhibitory substance that showed a bactericidal (Barrow, 1963) or a bacteriolytic (Arvidson *et al*., 1970) mode of action. In our case, the effect of staphylococcin 188 on growing and stationary phase cells of *M. luteus* was bactericidal, as the optical density of the cell suspension remains constant throughout the course of experimentation. Similarly, Bac1829 from *S. aureus* KS1829 was also a bactericidal peptide (Crupper and Landolo, 1997).

The present study demonstrates that *S. aureus* 188 produced a bacteriocin-like inhibitory substance i.e. staphylococcin 188 with a broad spectrum of antimicrobial activity directed against both inter and intra-generic Gram-positive indicator organisms. It can be used in future as chemotherapeutic agent.

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