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Efficacy of partial purified bacteriocin of *Pseudomonas aeruginosa* on Methicillin-resistant *Staphylococcus aureus* biofilm

Arumugam Thangarasu, Dhanam Selvam, Sampath Gattu, Kayalvizhi Nagarajan*

Department of Zoology, School of Life Sciences, Periyar University, Salem-636011, Tamil Nadu, India

ABSTRACT

Biofilms are microbial communities that cause serious chronic infections in the environment by enhancing antimicrobial resistance. Bacteria in the biofilm can be up to a thousand times more resistant to antibiotics than the same bacteria circulating in a planktonic state. The emergence of antibiotic-resistant microorganism has led to the exploration of different therapeutic agents like ribosomally synthesized microorganism peptides referred to as bacteriocins. In this study, bacteriocin producing bacteria *Pseudomonas aeruginosa* isolated from a soil sample. It was found to be effective against Methicillin-resistant *Staphylococcus aureus* (MRSA). Furthermore the bacteriocin was partial purified by ammonium sulfate, the precipitate has highly effective against MRSA (400AU/mL). MRSA cells were treated with precipitated culture supernatant of *P. aeruginosa* TA6 was analyzed by FT-IR. The treated and untreated MRSA showed band variations at 682.59 and 3442.15cm^{-1} corresponding to the alkyl and amide group respectively. Bacteriocin showed marked inhibition activity against the biofilm of MRSA. About 0.05% and 0.02% attachment of biofilm was observed in the presence of 1X MIC (10 µg/mL) and 2X MIC (20 g/mL) respectively. Our results recommend that bacteriocins that make stable pores on biofilm cells are extremely potent for the treatment of MRSA biofilm infections.

Keyword: *P. aeruginosa* TA6; Bacteriocin; MRSA; Biofilm; FT-IR

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***Address for Correspondence:**

Dr. Nagarajan Kayalvizhi, Assistant Professor, Department of Zoology, Periyar University, Salem - 636 011 Tamil Nadu, India.

INTRODUCTION

Biofilms are often associated with chronic disease states that create long-term problems for environment. Biofilms have been known to throw into physical and chemical protection as well as defense from antimicrobials[1]. In clinical settings, the continued existence of biofilms on medical devices and hospital equipment permits certain pathogens to easily transmit a disease to patients. Once infected, pathogen-associated biofilms can avoid human host immune defenses and are frequently associated with persistent infections, often resistant to antibiotic therapy[2]. Away from antimicrobial resistance, bacteria present in a biofilm are also defiant to various physicochemical stresses, enabling biofilms to persist in even the harshest of conditions. In the food industry, biofilms can cause food-borne disease outbreaks. Furthermore, inefficient cleaning commands may be a causative factor in the spread of resistance in hospital environments[3].

A recent comprehensive review described the efficacy of antibiotics in combination with other antimicrobial peptides and essential oils, as well as the effectiveness of biofilm-degrading enzymes, quorum sensing inhibitors and nanoparticles as potential antibiofilm agents[4]. Due to the extensive resistance of biofilms to conventional antibiotics, one alternative avenue to embark upon this problem is to harness bacteriocins as antimicrobials either independently or in combination with existing proven antimicrobials, to target biofilms. The major differentiation between bacteriocins and antibiotics is that bacteriocins restrict their activity to strains of species associated with the producing species and particularly to strains of the same species. Besides, bacteriocins are ribosomally synthesized and produced throughout the primary phase of growth, though antibiotics are usually secondary metabolites[5]. Bacteriocins usually have a low molecular weight (rarely over 10 kDa) they undergo posttranslational modification and can be easily degraded by proteolytic enzymes especially by the proteases of the mammalian gastrointestinal tract, which makes them secure for human consumption.

Bacteriocins are in general cationic, amphipathic molecules as they contain an excess of lysyl and arginyl residues[6, 7]. There are some reports available on bacteriocin-producing *Pseudomonas aeruginosa* isolated from soil samples but limited studies describe soil isolates[8, 9]. *P. aeruginosa* can compete, is by producing chromosomally encoded bacteriocins, called pyocins. Pyocins are ribosomally synthesized bacteriocins which are produced to kill competitors of the same species[10]. The pyocins produced by *P. aeruginosa* are classified into three major types: S, R- and F-pyocins[11]. R- and F-type pyocins are produced by more than 90% of *P. aeruginosa* strains, while 70% of *P. aeruginosa* strains can produce at least one S-type pyocin. The present study was partially purified bacteriocin extracted from *P. aeruginosa* TA6 culture showed biofilm activity against Methicillin-resistant *Staphylococcus aureus* (MRSA).

MATERIALS AND METHODS

Preparation and partial purification of Bacteriocin

The bacteriocin producing bacteria *P. aeruginosa* TA6 was grown in Luria broth and it was incubated at 37 °C for 18 hours. The following time, the culture broth was subjected to centrifugation at 10,000 rpm for 15 min at 4 °C for separation of bacterial cells. The supernatant was filter-sterilized by passing through a 0.45 µm pore sized filter membrane (Millipore, MA, USA). This CFS was further partially purified by ammonium sulfate precipitation[12]. To achieve the maximum saturation of bacteriocin, 70% of ammonium sulfate was added by constant agitation at 4 °C at overnight. Then precipitates were recovered by centrifugation at 10,000 rpm for 30 min at 4 °C. The resulting pellets were re-suspended in 20 mM sodium phosphate buffer (pH 7.2) and were designated as `partially purified bacteriocin'[13]. For removal of the salt from the partially purified bacteriocin, ultra-filtration was done through a pre-treated dialysis tubing of 6-8 kDa cutoff size[12]. The active fractions, thus obtained, were collected and pooled for assessment of inhibitory activity[14]. The bioactivity of bacteriocin was analyzed at each point of purification by agar well diffusion assay (AWDA), in terms of AU/mL. After partial purification, protein concentration was measured[15].

FT-IR Analysis

Determining the possible functional groups by FT-IR analysis was performed using Perkin Elmer to detect the characteristic peaks and their functional groups. The vibration pattern that appears in the infrared spectra provides information about the chemical functional group of the sample. 100 AU/mL^{-1} bacteriocin prepared and 10 × 0.6 OD indicator strain were mixed and incubated at 37 °C for 1h, 2h, and 3h. The control was used as without bacteriocin culture. A fraction of the sample was encased directly in the sample holder and spectra were scanned from 3442 to 682 cm^{-1}.

Biofilm Formation Assay

Methicillin-resistant *Staphylococcus aureus* was grown on Luria broth (LB; Himedia) at 37 °C for 18 h. 200 µL of bacterial cell suspension (about 10^6 CFU/mL) in Luria broth (LB) were inoculated in triplicates to wells of a tissue culture polystyrene 96-well plate. Biofilms were developed for 48 h at 37 °C. Subsequently, the medium was removed and the biofilms were washed twice with 250 µL of sterile phosphate-buffered saline (PBS, pH 7.2). Biofilms were fixed with 200 µL of methanol per well for 15 min and stained with 200 µL of 1% crystal violet per well (Sigma-Aldrich, Steinheim, Germany). After that, the plates were rinsed with distilled water and air-dried. Crystal violet was solved in 96% ethanol to measure absorbance at 492 nm in a microplate reader (Bio-Rad, USA). Each assay was performed three times and the results were averaged. Values of absorbance <0.2 were considered to be weak producers, 0.2–0.4 were medium producers, and values >0.4 were considered strong producers[16].

RESULTS

Partial purification of bacteriocin from a soil isolate *Pseudomonas aeruginosa* TA6

The crude bacteriocin preparation obtained from the cell-free supernatant of *P. aeruginosa* TA6 after ammonium sulfate saturated precipitation (70%) was subjected to purification. The ultrafiltration of the crude bacteriocin was achieved by using dialysis tubing of cut off size 6-8 kDa. The bacteriocin activity was carried against Methicillin-resistant *Staphylococcus aureus* (MRSA) by agar well diffusion assay. The maximum antagonistic activity was observed to be 400 AU/mL in the resolved partial purified bacteriocin. (Fig 1)

Fig 1. Partially purified bacteriocin against MRSA.

Fourier Transmission-Infra Red Analysis (FT-IR)

The FT-IR spectral analysis of the extracellular extract of *P. aeruginosa* TA6 strain revealed that the spectral range of obtained functional group ranged was between 400 and 4000 cm^{-1} from the results, it was observed that the peak signal recorded for 682.59,1046.13, 1103.95, 1165.28, 1638.22, 2078.63, 2931.77 and 3442.15. The peak observed at 682.59, was due to the alkyl group (C–Cl). The vibration stretch recorded at 1046.13 aromatic groups (C-Br), 1103.95 aromatic group (C-F), 1165.28 aromatic group (C-F). The sharp peak at 1638.22 cm^{-1} was due to the presence of N, N-disubstituted Amides. The small vibration stretch recorded at 2078.63 cm^{-1} represents the possible presence of thiocyanate (–N=C=S). Finally broadband at 3442.15 cm^{-1} showed the presence of amide group (–CONH–) (Fig.2)

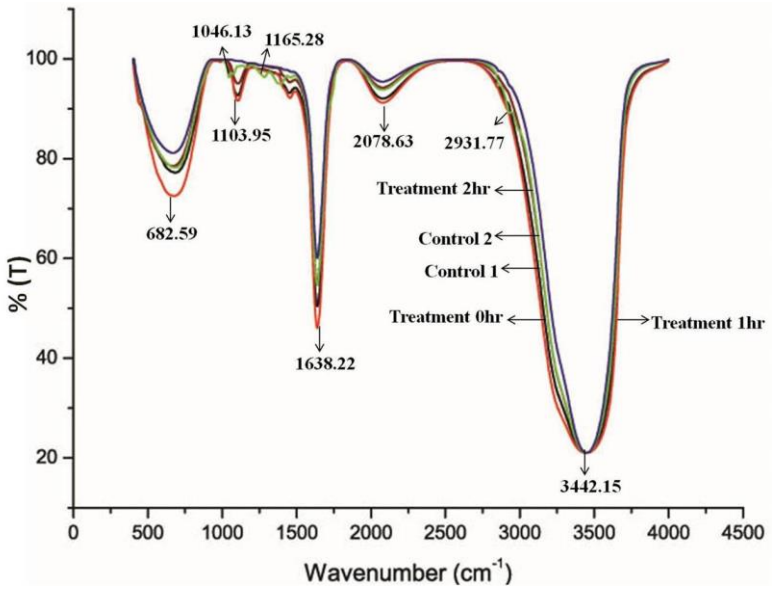

Fig 2. Bacteriocin functional group identified by FT-IR analysis.

Biofilm inhibition by bacteriocin.

When different concentrations of bacteriocin were incubated with Methicillin-resistant *S. aureus* for 4 h at 37 °C for adherence to the wells of microtiter plates, it inhibited biofilm attachment in a concentration-dependent manner. About 0.05% and 0.02% attachment of biofilm was observed in the presence of 1X MIC (10 µg/mL) and 2X MIC (20 µg/mL) respectively. Bacteriocin showed significant inhibitory activity against Methicillin-resistant *S. aureus* biofilm formation at 48 h, about its concentration. For the non-treated controls, a biofilm formed consisted of nearly uniform, a thick layer of cells. While the biofilm treated with bacteriocin 10 µg/mL and 20 µg/mL was much less dense, and individually formed colonies could be seen (Fig. 3).

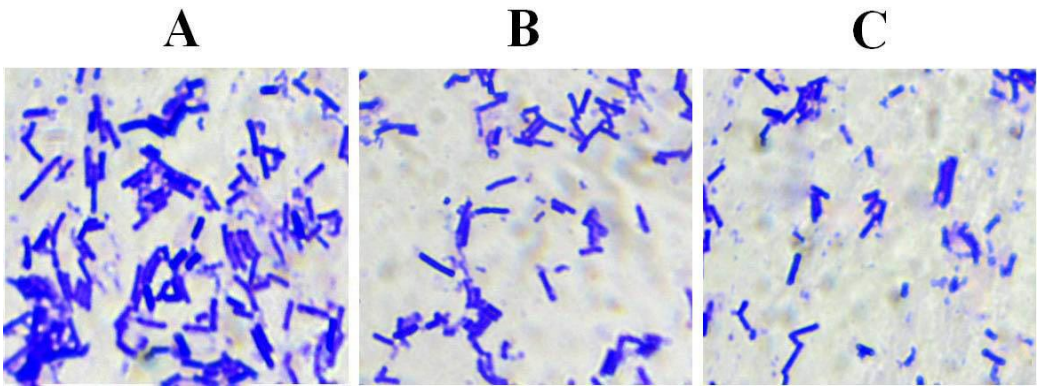

Fig 3. Biofilms treated with bacteriocin assessed by microscopy: A. non-treated control; B. Treated with bacteriocin 1X MIC (10 µg/mL); C. Treated with bacteriocin 2X MIC (20 µg/mL).

DISCUSSION

The growth of biofilms is a significant problem within the healthcare and food industries. Control of biofilms formed by microbial pathogens is an important subject for medical researchers since the development of biofilms on foreign-body surfaces often causes biofilm-associated infections in patients with indwelling medical devices. The characteristic resistance offered by biofilm-associated communities of microorganisms leading to their persistent survival is an important challenge to address. Though, many antibiotics are effective against biofilms[17]. Furthermore, while it is suggested that bacteriocins may inhibit the development of biofilms[18], their effect upon microbial cells in a biofilm is not fully understood. Methicillin-resistant *Staphylococcus aureus* (MRSA) is an alarming threat of interest that is responsible for either community-acquired (CA-MRSA) or health-care-acquired (HA-MRSA) infections. *Staphylococcus aureus* is an opportunistic pathogen causing a broad range of nosocomial and community-acquired infections. Diseases caused by this bacterium can range from skin infections to foodborne illnesses and severe infections such as endocarditis, osteomyelitis, and sepsis[19]. The continuous use of antibiotics has resulted in multiresistant bacterial strains all over the world[20]. Consequently, there is an urgent need to search for alternatives to synthetic antibiotics.

Bacteriocins are the peptides and protein antibiotics which are produced by several species and have antimicrobial properties usually against other closely related species[21]. There are some reports available on bacteriocin-producing *P. auroginosa* isolated from soil samples but limited studies describe soil isolates[8, 9]. Bacteriocins of *P. aeruginosa* present an example of such excellence as >90% of its strains produce bacteriocins called Pyocins[10, 22, 23]. In this study, the effects of bacteriocin from *P. aeruginosa* TA6 against Methicillin-resistant *staphylococcus aureus* (MRSA). Among the bacteriocins, showed the highest bactericidal activities

against biofilm cells. The partially purified bacteriocin has shown the highest activity (400AU/mL) against MRSA. The FT-IR spectrum of *P. aeruginosa* TA6 bacteriocin treated with MRSA. The strain was revealed that functional range at 300 and 4000 cm^{-1}. In bacteriocin treated cells shift in absorbance in low frequency at 682.59, 1046.13, 1103.95, 1165.28, 1638.22, 2078.63, 2931.77 and 3442.15 cm^{-1}. FT-IR spectroscopy has been applied as a reliable method to study the putative mode of action of cell lytic bacteriocins from *P. aeruginosa* on *S. aureus.* However, our result was contradicted the earlier report[24]. The fall in the emergence of new antimicrobials in the market during the past two decades is worrying, particularly given the rise in bacterial resistance against many of the currently used antibiotics. In addition to antimicrobial activities, bacteriocins serve as an anti-resistance compound to classic antibiotics as they can interact with bacterial membranes, create ion-permeable channels leading to increased cytoplasmic membrane permeability and hence, bacterial cell death[25]. When applied to preformed MRSA biofilms, bacteriocin was able to significantly reduce biofilm cell viability even at lower concentrations at 24-h incubation. These results suggest that bacteriocin is controlling for the growth of MRSA biofilm. Our results suggest that bacteriocins that form stable pores on biofilm cells are highly potent for the treatment of MRSA biofilm infections.

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