IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF BACTERIA HAVING ANTIMICROBIAL AND ANTIBIOFILM ACTIVITY

GARIMA SHARMA, SHWETA DANG, SANJAY GUPTA, REEMA GABRANI*
Department of Biotechnology, Jaypee Institute of Information Technology, A-10, Sector 62, NOIDA, India
Email: reema.gabrani@jiit.ac.in

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ABSTRACT

Objective: The aim of the current study was to isolate and identify the bacteriocinogenic strain exhibiting broad range antimicrobial activity and antibiofilm activity from the soil of animal farms.

Methods: In the current study, bacterial strains were isolated from soil of twelve different regions of the animal farm all over India and screened for antimicrobial activity against *Staphylococcus epidermidis*, *Micrococcus luteus*, *Pseudomonas fluorescens* and *Escherichia coli*. Antibiofilm ability of these selected strains was checked on preformed biofilm of *S. epidermidis* and in addition biofilm disruption potential was also determined. The potent bacterial strain was identified at the molecular level by 16S ribosomal DNA (rDNA) sequencing.

Results: 30 out of 231 strains isolated from soil were selected on the basis of antibacterial activity against *S. epidermidis*. One potential candidate (GAS 101) exhibited ≥99% inhibition against *S. epidermidis*, *M. luteus*, *P. fluorescens* and *E. coli* and also showed antibiofilm activity. GAS 101 16S rDNA sequencing data identified it as *Bacillus subtilis*. The sequence of *B. subtilis* was submitted to genbank under accession no. KJ564301.

Conclusion: *B. subtilis* GAS 101 isolated from soil of animal farm showed the antibacterial activity against all indicator organisms and also displayed antibiofilm activity against preformed biofilm and inhibited biofilm formation of *S. epidermidis*.

Keywords: *B. subtilis*, *E. coli*, *M. luteus*, *P. fluorescens*, *S. epidermidis*

INTRODUCTION

Bacterial nosocomial infections can pose a major risk mainly in immune-compromised patients. Advancement in medical sciences has led to the increased use of invasive devices resulting in high rates of infection [1]. Bacteriocins are ribosomally synthesized peptides which bacteria use as their natural defense mechanism in the same niche area. Bacteriocins have many properties like low toxicity, potency, narrow and broad spectrum effectiveness and in situ production. These properties indicate that bacteriocin can be used as alternate to antibiotic [2]. Production of bacteriocin is widespread in nature; a single bacterial species has been reported to produce 10 to 100 different types of bacteriocins [3]. Lactic acid bacteria (LAB) and *Bacillus* species both have gained the focus because of the ability to produce broad-spectrum inhibitory activity and GRAS status by the US FDA. Nisin and pediocin are the most studied antimicrobial substance from LAB which are commonly used in food production industry as a preservative agent and in the pharmaceutical industry as a health care product [4, 5].

An antimicrobial substance produced by *Bacillus* species may be placed on the second place after LAB because of the diversity in the antimicrobial peptide (AMP) due to different chemical structures [6]. The *Bacillus* subgroup has been reported to produce a large number of AMPs and this can be a good source to explore the novel antimicrobial substances [7, 8]. Subtilin synthesized by *B. subtilis* is a cationic AMP which belongs to lantibiotic and has been studied extensively. It has been shown to be active against broad spectrum gram positive bacteria and acts via pore formation [9]. A number of reported bacteriocins is very less as compared to the reported bacterial species, so necessitating a need to explore the properties and therapeutic applications of bacteriocins. The objective of the current study was to isolate the bacteria producing antimicrobial substance from soil of animal farm. The soil is a rich source of microorganisms, as it provides an enhanced environment for the growth of microbes, which contributes towards enormous diversity of bacteria. In the current study, we have isolated different microbes which are able to produce antimicrobial substances from the soil of animal farm using de Man, Rogosa and Sharpe (MRS) agar plates. The isolate that possessed the broad activity against indicator organisms and was able to disrupt biofilm was further identified by molecular characterization.

MATERIALS AND METHODS

Chemicals
The Chemicals and media used in the current study were procured from Hi-Media, CDH and Sigma-Aldrich.

Methods
Bacterial strains and growth conditions
Four different microorganisms *S. epidermidis* (MTCC no. 435), *P. fluorescens* (MTCC No. 2421), *M. luteus* (MTCC No. 106) and *E. coli* (MTCC No. 443) used in the current study were procured from MTCC, Chandigarh. All bacteria were cultured in nutrient broth at 37 °C for 16-18 h.

Isolation of antimicrobial substance producing bacteria from soil of animal farm
Bacterial strains were isolated from soil collected near the dairy of the various region of India (table 1). One gram of soil was mixed with 9 ml of autoclaved distilled water, homogenized using a vortex mixture and serially diluted with autoclaved water. Subsequently, 100 μl of each dilution was spread onto MRS agar plate (Hi-media) and the plates were incubated at 30 °C for 24 h. Well-isolated colonies were inoculated into fresh MRS broth for stock preparation.

Inhibition spectrum of cell free supernatant (CFS) of isolated bacterial strains
The inhibition spectrum of each isolate was examined using the 96 well microtitre plate assay against indicator organisms. The supernatant was obtained by centrifugation (10,000 xg, 15 min at 4 °C) of culture grown in MRS broth at 30 °C for 24 h. CFS from overnight grown culture was incubated with the 1x10⁶ cfu/ml of the indicator organisms in each well of the 96 well plates [10]. Three wells in each

Original Article

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plate served as sterility control (without inoculum). Plates were incubated at 37 °C for 16-18 h and then examined for the growth inhibition with respect to control (indicator organism without CFS). The assay was carried out in triplicates and repeated thrice.

Determination of activity unit per ml (AU/ml)

Antimicrobial activity (AU/ml) was expressed as the reciprocal of the highest dilution that gave a definite zone of inhibition multiplied by a dilution factor [11]. 1x10^5 cfu/ml of S. epidermidis was treated with the 1:2:1:28 dilution of the CFS from each isolate and incubated at 37 °C for 16-18 h. The highest dilution showing inhibition activity was considered as antimicrobial activity unit.

Antibiofilm activity of cell-free supernatant

CFS from each isolate was tested for its potential antibiofilm activity against preformed S. epidermidis biofilm and also on its biofilm formation.

Inhibition of cell attachment

CFS from each isolate was incubated with 1x10^6 cfu/ml of S. epidermidis in the 96 well plate and incubated at 37 °C for 48 h to allow cell attachment and biofilm development. The plate was washed with phosphate buffer saline (PBS) and the crystal violet staining assay was performed [12]. Evaluation of % reduction in biofilm was evaluated by-

\[
\%\text{ inhibition} = \left[1- \frac{\text{absorbance of treated bacteria}}{\text{absorbance of untreated bacteria}}\right] \times 100
\]

Reduction of biofilm growth and development

The plates containing 1x10^5 cfu/ml of S. epidermidis were incubated at 37 °C for 48 h to allow cell attachment and biofilm development.

Table 1: Soil samples collected from different regions of India

| Sample no. | Collection site | Total isolates | Isolates with antimicrobial activity |
|------------|----------------|----------------|------------------------------------|
| 1          | Ghaziabad (U.P.) | 26             | 1                                  |
| 2          | Ghaziipur (Delhi) | 33             | 10                                 |
| 3          | Gurgaon (Haryana) | 12             | 2                                  |
| 4          | Bijnor (U.P.) | 19             | 4                                  |
| 5          | Agra (U.P.) | 17             | 3                                  |
| 6          | Mathura (U.P.) | 35             | 9                                  |
| 7          | Bulandshahr (U.P.) | 17      | 1                                  |
| 8          | Faridabad (Haryana) | 22       | 0                                  |
| 9          | Dehradun (Uttaranchal) | 24     | 0                                  |
| 10         | Sirdi (Maharashtra) | 14       | 0                                  |
| 11         | Bhopal (M.P.) | 17             | 0                                  |
| 12         | Sikar (Rajasthan) | 12             | 0                                  |
| Total      |                 | 231            | 30                                 |

Antimicrobial spectrum of cell free supernatant of selected strains

Antimicrobial activity of screened bacterial CFS was examined further against P. fluorescens, M. luteus and E. coli indicator organisms by 96 well microtiter plate assay to select the isolate possessing the broad range antimicrobial activity. The results of checker board assay indicates that 28 out of 30 strains showed >90% inhibition against E. coli, whereas 20 out of 30 showed inhibition of P. fluorescens cells and 19 strains were active against M. luteus (table 2). In totality 10 strains showed >90% inhibition against all chosen bacteria.

Determination of AU/ml

The AU/ml of CFS from all selected bacterial strains was checked against S. epidermidis as indicator organism (table 2). The activity ranged from 80 to 800 AU/ml and the highest activity was observed for GAS 101 strain up to 800AU/ml against S. epidermidis.

Antibiofilm activity of cell free supernatant

The antibiofilm activity of all the selected strains (30) after initial screening was checked against S. epidermidis biofilm. The capability of S. epidermidis to produce biofilm was checked on the congo red agar (data not shown). S. epidermidis is an opportunistic dermal pathogen which can cause infections on indwelling medical devices and surgical wounds by forming biofilm [15]. The capability of S. epidermidis to form biofilm complicates its removal as it is more resilient to eradication [16]. In the current study, the curing i.e. biofilm disruption as well as the preventive prospective, especially inhibition of biofilm formation, was investigated. Ten out of 30 chosen strains were potentially able to inhibit >90% S. epidermidis biofilm formation upon CFS incubation (table 2) and 4 strains could demolish the preformed biofilm by S. epidermidis.

Molecular characterization of potential candidate

The isolate which displayed antimicrobial activity against all selected strains, antibiofilm activity (formation and disruption) against S. epidermidis and demonstrated highest AU/ml was selected for molecular characterisation. The 16S ribosomal DNA was amplified by 16S universal primers and its DNA was sequenced for strain identification. The strain (GAS 101) was identified as B. subtilis strain and 16S rDNA partial sequence was submitted to GenBank (accession no. KJ564301). The phylogenetic tree of the amplified sequence of 16S rDNA of B. subtilis strains GAS101 with closely related Bacillus species was constructed by CLUSTAL W software.
Soil typically contains $10^5$ to $10^8$ microorganisms per gram [dry weight] which may represent more than a million bacterial species [17]. Soil from animal farm remains rich in straw, dung urine and traces of raw milk, all of these provide an absolute atmosphere to cultivate. Serially diluted soil sample was allowed to grow on the MRS agar plate. MRS agar is a selective medium containing sodium acetate which suppresses the growth of other competing bacterial group and favours the growth of lactic acid bacteria group and Bacillus species [18]. In preliminary selection, S. epidermidis was used as an indicator organism which is an opportunistic pathogen reported for causing infection. In the current study 30 isolates out of 231 showed >90% inhibition against S. epidermidis and were further screened for broad range antibacterial activity. Other indicator organisms used in the current study belong to gram positive (M. luteus) and gram negative (E. coli and P. fluorescens) clan and have been reported to cause nosocomial infection [16, 19-21]. These infections persist and are resilient to antibiotic treatment. Ten strains showed >90% inhibition against indicator organisms in the current study which indicates towards the broader range of growth restriction. Nisin, Type A (1) laeticibactin, is FDA approved and has GRAS status which can restrict the growth of both gram positive and gram negative disease causing bacteria. Though nisin is approved as food biopreservative, it has proved its potential for other biomedical applications as well [22]. Apart from LAI, antimicrobial substance from B. subtilis has also been reported against the broader range of bacteria inclusive of Salmonella, Bacillus cereus and Staphylococcus aureus [23]. B. subtilis can produce wide array of antimicrobial substances having varied biochemical and biological properties.

S. epidermidis has the ability to colonize on the hospital device and form biofilm. Biofilm is multicellular, surface-attached film which provides architecture to microorganisms that contribute to resistance to antibiotics [16]. In the current study, two strains namely GAS 101 and GAS 301 demonstrated broad range antibacterial activity and in addition were able to inhibit S. epidermidis biofilm formation and showed disruption of biofilm. The molecular identification of strain GAS 101, with broader range of activity against S. epidermidis was reported by Gabrani et al. [24]. BLIS extracted from B. subtilis BS 15 against B. cereus showed 100 AU/ml [25].

The chance of developing resistance to bacteriocins due to their diverse mechanism of action is less as compared to antibiotics [26]. Despite the huge potential, there are few reports available where...
antimicrobial substances obtained from B. subtilis have been targeted against biofilm. Members of Bacillus group are considered good producers of diverse antimicrobial substances, including peptide, lipopeptide antibiotics and bacteriocins [6]. Bacitracin from Bacillus species are of interest because of their broader range of activity including Gram negative bacteria and fungi [27]. Bacitracin like inhibitory substance from Bacillus species has been reported for its antimicrobial activity and antibiofilm potential [28]. Sonorensin, a bacteriocin isolated from marine isolate Bacillus sonorenensis, has exhibited S. aureus biofilm inhibition and since it could target both multiplying and non-multiplying bacteria, the resistance could not be developed against it [29]. Lipopeptides isolated from B. subtilis have shown the reduction in biofilm formation by 88% and dispersion of mature biofilm by 81% [30].

CONCLUSION

Aim of the current study was to isolate the strain with antimicrobial and antibiofilm potential against causative agents of nosocomial infections. Soil samples were collected from animal farm of twelve different regions. Total 231 isolates were preliminary screened against S. epidermidis and 30 isolates exhibiting more than 90% antimicrobial activity were selected. Selected strains were checked for their broader range of activity and the antibiofilm potential against S. epidermidis biofilm. Only one potential strain out of 30 showed >99% inhibition against all indicator organism with highest activity unit (800AU/ml) on S. epidermidis and antibiofilm potential against causative agents of nosocomial infections due to multidrug-resistant strains. The selected strains were further checked for their antibiofilm potential against causative agents of nosocomial infections due to multidrug-resistant strains.

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CONFLICT OF INTERESTS

There is no conflict of interest.

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