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Study on antagonistic activity of a novel bacterial isolate under mild stress condition of certain antimicrobial agents

***Khusro A.[1], Preetam Raj J. P.[1] and Panicker S. G.[1,2]**

[1]*Department of Plant Biology and Biotechnology, (PG. Biotechnology), Loyola College, Nungambakkam, Chennai (India)*
[2]*Helen Keller Research Centre (HKRC), Loyola College, Chennai (India)*

ABSTRACT

Bacteria launch stress response in adverse and unfavourable environmental conditions. Antimicrobials or antimicrobial agents are one of the stresses for the microorganisms. The aim of the present investigation was to determine the antagonistic activity of a novel bacterial strain under normal and stressed conditions against some of bacteria including human pathogens. The identification of bacterium as Bacillus subtilis strain KPA was confirmed by subjecting its amplicon (483 bp) to 16S rRNA gene sequence analysis and pairwise alignment through BLAST tool. A definite volume of antimicrobial agents such as Allium sativum, ampicillin and Mercuric chloride at their sub- MIC values was added to the lag phase culture of strain KPA in order to provide stress to bacteria. The antibacterial activity of strain KPA (both control and stressed) against Staphylococcus aureus, E.coli, Bacillus subtilis, Proteus vulgaris and Staphylococcus epidermidis was determined through agar well diffusion method. The extracellular proteins or peptides secreted by strain KPA, both in normal (control or non-treated) and stressed conditions (treated), were found to be ineffective against these microbes. The present study clearly indicates that antagonistic nature of the bacteria depends upon the type of strain, source of isolation and their genetic organization.

Key words: Antimicrobials, Antagonistic activity, Agar well diffusion, *Bacillus subtilis*, sub- MIC

INTRODUCTION

Bacillus genus is a group of Gram- positive, aerobic and endospore forming rod- shaped bacteria. *Bacillus* bacteria are among the most widespread microorganisms in nature. *Bacillus* sp. is a known producer of antimicrobial substances such as peptides, antibiotics and bacteriocins. These bioactive substances have major applications in various industrial areas. One of the most important species of genus *Bacillus* is *Bacillus subtilis* that can survive in extreme conditions because of the production of endospores. *Bacillus subtilis* is non- pathogenic and non- toxigenic to humans, animals and plants [1]. Bacteria of *Bacillus* genus are known to produce more than 200 antibiotics. *Bacillus* antibiotics differ in their structure as well as spectrum of activity [2]. Antibiotics play an important role in regulating the microbial populations of soil, water and sewage. *Bacillus* genus are well known producer of antibiotics as secondary metabolites [3, 4]. Some strains of *Bacillus* synthesize bacteriocins, which are only effective against bacteria of the same species, others produce antibiotics against Gram- negative bacteria and still other strains

have a wide spectrum of antibiotic activity [Stein, 2005]. Bacteriocins are microbial compounds that have a bactericidal or bacteriostatic effect on other closely related species [5]. The ability of *Bacillus subtilis* to secrete grams per litre of proteins directly into the growth medium has also made them prime producer of heterologous proteins. In recent years, many studies have been emerged in order to investigate the antimicrobial properties of strain of *Bacillus* [6, 7] but still there are very few reports on the antagonistic activity of *Bacillus subtilis* against human pathogens under mild stress condition. In view of this the present context was investigated to determine the antibacterial activity of a novel strain of *B. subtilis* against few bacteria under mild stress condition of various antimicrobial agents.

MATERIALS AND METHODS

Sample collection, Isolation and Screening

Poultry faeces sample was collected from poultry farm of Guduvanchery, Tamil Nadu (India). Faeces sample was brought to the laboratory in aseptic condition. A serial dilution of the sample (1 g of faeces soil) was made using sterile saline until a dilution of $10^{-6}$was obtained. Hundred microlitre of this dilution was spread over nutrient agar petriplates and incubated at 37ºC for 24 hours. Pure culture was isolated and subcultured in the same medium at 37ºC. The culture was streaked and kept in incubator at 37°C for 24 hours and was preserved in slants at 4±2°C.

Morphological and Biochemical tests

Purified isolate was characterized by Biochemical analysis using Indole test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Catalase test, Urease test, Oxidase test and Amylase test (according to the Bergey's Manual of Systemic Bacteriology). Gram staining, Endospore staining and Motility test were performed under Morphological tests.

Isolation of Genomic DNA

Two ml of bacterial culture were centrifuged at 6000 rpm for 5 minutes. The supernatant was discarded. One ml of UniFlex^{TM} Buffer 1 and 10 µl of RNase were added to the pellet obtained. Mixed well by pipetting and incubated for 30 minutes at 37ºC in a water bath. To the lysed samples 1 ml of 1:1 phenol: chloroform was added and mixed well. The samples were centrifuged at 10,000 rpm for 15 minutes at room temperature. The aqueous layers were separated in a fresh 1.5 ml vial. To the aqueous layer 1 ml of UniFlex^{TM} Buffer 2 were added and mixed well by pipetting. The mixture was centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant was discarded. To the pellet 500 µl of 70% ethanol were mixed. Again it was centrifuged at 10,000 rpm for 10 minutes at 4ºC. The supernatant was discarded. The pellet was air dried for about 10-15 minutes till the ethanol evaporates. The pellet was resuspended in 50-100 µl of UniFlex^{TM} Elution Buffer. DNA was stored at -20ºC.

Amplification of 16S rRNA genes by PCR, Sequencing and Alignment

The 16S ribosomal RNA was amplified by using the PCR (ependorfep.Gradient) with *Taq* DNA polymerase and primers 27F (5` AGTTTGATCCTGGCTCAG 3`) and 1492R (5`ACGGCTACC TTGTTACGACTT 3`). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and visualized by alpha image gel doc after ethidium bromide staining. The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and USA). The same primers as above were used for sequencing. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at http:// www.ncbi-nlm-nih.gov/.

Test cultures

Staphylococcus aureus, *E.coli*, *Bacillus subtilis*, *Proteus vulgaris* and *Staphylococcus epidermidis* were obtained from Department of Plant Biology and Biotechnology, Loyola College, Nungambakkam (Tamil Nadu). The bacterial cultures were preserved at 4ºC for further investigation.

Antimicrobials of interest and their preparation

Allium sativum (Spice), ampicillin (Antibiotic) and Mercuric chloride (Heavy metal) were used to provide stress to the novel bacterial strain. *Allium sativum* (Garlic) was purchased from local market of Nungambakkam, Tamil Nadu (India). The garlic bulbs without the outer skins were grinded in a sterilized mortar and pestle. The fine garlic mesh

was centrifuged at 6000 rpm for 10 minutes. The supernatant was filter sterilized by using a 0.2 µm syringe filter to produce sterile supernatant. Heavy metal salt solution was prepared by mixing Mercuric chloride ($HgCl_2$) in sterilized distilled water at the concentration of 25 mg/L. Ampicillin (10 µg) was prepared by mixing appropriate volume of DMSO.

MIC and sub- MIC determination of antimicrobials

MIC and sub- MIC values of *Allium sativum* juice, Mercuric chloride and ampicillin were determined by Microdilution method [8]. Serial dilutions of *Allium sativum* juice, Mercuric chloride and ampicillin were prepared from 100% to 1% concentration. The highest dilution of antimicrobials inhibiting the bacterial growth was considered as MIC value. Half of the value of MIC was considered as sub- MIC value.

Shake flask fermentation

The fermentation was carried out in conical flasks (volume capacity 250 ml), each flask containing 50 ml of Nutrient Broth. One flask was kept as control (no addition of antimicrobial agents). Rest of the flasks was labelled as *Allium sativum*, ampicillin and Mercuric chloride. The flasks were sterilized at 121ºC for 15 minutes. Each flask was inoculated with 500 µl of overnight bacterial inoculum. The flasks were kept in the rotatory shaker at 37ºC for 2 h (lag phase of novel strain). The flasks were taken out and each flask except control was inoculated with appropriate volume of antimicrobials (from sub-MIC value) as labelled on the flasks. All the flasks were again kept in rotatory shaker for 48 h of incubation.

Detection of antagonistic activity

The bacterial cultures grown after 48 h of incubation were centrifuged at 8000 rpm for 10 minutes at 4ºC. The supernatants were collected and filter sterilized using 0.2 µm syringe filter. The antagonistic activity of novel bacterial strain against the tested human pathogens was determined through Agar well diffusion method. The broth culture of each tested bacteria was spread over the Nutrient agar plates using sterile cotton swabs. Wells were created on agar plates with 5 mm cork borer. Hundred microlitres of supernatants from novel bacterial isolate (control and stressed bacteria) were poured in the wells. The antimicrobials at sub- MIC values were poured in the wells as negative control. The plates were incubated right side up at 37º C and the zone of inhibition was observed after 24 h.

RESULTS AND DISCUSSION

Isolation and identification of new strain of bacteria

The isolated bacterial strain was identified as *Bacillus* sp. based on morphological and biochemical characteristic. Genomic DNA of the isolate was visualized under UV. The amplicon of 483 bp was observed using PCR amplification. In the present study, 16S rRNA gene sequencing of the isolate was investigated. The isolate was identified as *Bacillus subtilis* strain KPA by comparing the similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST. The comparison showed that the similarity of 16S rRNA gene sequences was 99%. The identities of strain KPA were determined by comparing them with the available sequences of the strains and with high scored rRNA sequences in BLAST search. The novel isolated sequence was deposited in Genbank (Accession number-KC918878), maintained by NCBI, USA.

MIC and sub-MIC determination of *Allium sativum,* ampicillin and Mercuric chloride

The results from Table 1 indicated that the MIC values of *A. sativum*, ampicillin and Mercuric chloride on *B. subtilis* strain KPA was 10%, 10% and 20% respectively. The sub-MIC values (which is 0.5 x MIC) for each treatments was further calculated as 5%, 5% and 10% for *A. sativum*, ampicillin and Mercuric chloride respectively.

Antagonistic activity

The effect of extracellular proteins and peptides secreted by strain KPA in normal and stressed conditions on few bacteria was observed after 24 h of incubation. Peptides and antibiotics secreted by strain KPA (control) were found to be ineffective against the tested bacteria. On the other hand strain KPA under mild stress condition of *Allium sativum*, ampicillin and Mercuric chloride was also not showing inhibitory action against the test organisms. No zone of inhibition was observed by the negative controls against the tested bacteria.

Table-1: Shows MIC and sub- MIC values for antimicrobials against strain KPA

Antimicrobials	MIC value (%)	Sub- MIC value (%)
Allium sativum	10	5
Ampicillin	10	5
Mercuric chloride	20	10

Table-2: Shows Antagonistic activity results of strain KPA against human pathogens

Bacteria (control and stressed)	Test organisms				
	Staphylococcus aureus	*E.coli*	*Bacillus subtilis*	*Proteus vulgaris*	*Staphylococcus epidermidis*
B.subtilis strain KPA	-	-	-	-	-
Strain KPA+ *A.sativum*	-	-	-	-	-
Strain KPA+ ampicillin	-	-	-	-	-
Strain KPA+ Mercuric chloride	-	-	-	-	-
Negative controls	-	-	-	-	-

'-'indicates No Zone of Inhibition

Bacillus genus is widespread in the environment being found in dust, soil, water and air. *Bacillus* bacteria are known to be effective antagonists of different pathogens. In recent years *Bacilli* were extensively studied as probiotics, due to their health benefits on the host [9, 10]. In the present study a novel strain of *Bacillus subtilis* was isolated from poultry farm and its antagonistic activity was determined against some of the bacteria such as *Staphylococcus aureus*, *E.coli*, *Bacillus subtilis*, *Proteus vulgaris* and *Staphylococcus epidermidis*. Inhibition of *Staphylococcus aureus* by *Bacillus* cultures was shown by other authors [11-13], but no anti-*Staphylococcus* effect was found in our finding. Strain KPA was found to be ineffective against *Staphylococcus* sp. *Bacillus subtilis* strain KPA, even after the exposure of different stress conditions, were not able to show antibacterial activity against *Staphylococcus* sp. Pinchuk *et al*. [14] reported that *Bacillus* strains have antibacterial activity against *E. coli*. Our reports were against the finding of Pinchuk *et al*. [2001] but the present investigation favours the finding of Perez *et al.* [15] who demonstrated that *B. subtilis* MIR 15 strain did not show antibacterial activity against *E. coli.* In a study on the antibacterial activity of 29 *Bacillus* strains isolated from the soil against some tested bacteria, Yilmaz *et al*. [16] determined that only 5 isolates had antibacterial activity. They concluded that not all the *Bacillus* isolates showed inhibitory effects on *E. coli.* The present study clearly indicates that the novel bacterial strain under normal and stressed condition was not showing antagonistic activity against *E.coli*. *B. subtilis* produce various biosurfactants, which have a high potential for biotechnology and pharmacology applications [17]. These compounds vary in structure and spectrum of activity and usually are responsible for antimicrobial effects of *Bacillus* bacteria [18]. In our study *B. subtilis* strain KPA were not able to produce active inhibitory compounds against the microbes tested here after the exposure of certain antimicrobials. This may be due to the reason that the antimicrobials were not able to 'switch on' the genes, responsible for the production of potential antibiotics and peptides which may inhibit the growth of the microbes tested here. The protein produced by *B. subtilis* ATCC 21332 after treating with 0.5 x MIC *A. sativum*, showed high antibacterial activity against *E. coli*. As a control, bacteria without treating with antimicrobial agents were also tested for antimicrobial activity. There was no inhibitory effect shown towards *B. subtilis* [19]. In the present investigation strain KPA was also not showing any activity against *Bacillus subtilis*. The commercially available antibiotics have the potential to kill both the pathogenic and non-pathogenic organisms. As *Bacillus subtilis* is industrially important organism so the present finding led to the generalized assumption that extracellular proteins or peptides secreted by strain KPA compared to commercially available antibiotics, may promote the growth of *B. subtilis* by showing symbiotic relationship.

CONCLUSION

In the present study *Bacillus subtilis* strain KPA was characterized for their antagonistic activity against *Staphylococcus aureus*, *E.coli*, *Bacillus subtilis*, *Proteus vulgaris* and *Staphylococcus epidermidis*. The novel isolate was found to be ineffective against the microbes tested here. This study also described that strain KPA in mild stress condition of certain antimicrobials was not able to inhibit other microbial growth. Further study of compounds, secreted by *Bacillus subtilis* strain KPA, will result in better understanding of the mechanisms of antagonistic activity of this particular strain. Another study should also be continued to isolate and identify novel strains having broad spectrum of antagonistic activity against these test organisms, which may play a major role in the area of Biotechnology and Pharmacology.

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