Plasmid borne BAC-IB17: Localization of a potential antibacterial positive marker (Bac$^+$) encoded broad inhibitory spectrum bacteriocin

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Abstract: Production of antimicrobial compounds is considered as ubiquitous anti-competitor strategy in bacterial ecosystem. Bacteriocins are heterogeneous; highly specific and efficient anti-competitor agents and the gene responsible for the production of bacteriocins mostly exist in an autosomal state and associated with plasmids. BAC-IB17 is a broad spectrum bacteriocin and its production was observed at different stages of the growth cycle from *Bacillus subtilis* KIBGE-IB17. Growth kinetics of *B. subtilis* KIBGE-IB17 along with the production of BAC-IB17 showed that it exhibited secondary metabolite kinetics. Plasmid curing technique revealed that the gene responsible for the bacteriocinogenecity in *B. subtilis* KIBGE-IB17 was located on the plasmid of the bacterium. Overlay method also demonstrated the plasmid-mediated bacteriocinogenesis of the isolated colonies. With the advancement in genomics and proteomics, the plasmid borne BAC-IB17 can play a significant role in the transfer of bacteriocinogenic factor to other incompetent cells and also in the maintenance of plasmid in bacterial population.

Keywords: *Bacillus subtilis*, antibacterial agent, bacteriocin, plasmid, secondary metabolite.

INTRODUCTION

Microorganisms are able to produce an astonishing range of defense systems and they invest substantial vigor for the production and establishment of various antagonistic agents. Numerous bacteria and fungi are capable of producing different extracellular toxic compounds beside other enzymes and metabolites. Toxic compounds from any microorganisms can act as a lytic agent and can easily lyse cell membrane of phagocytes by forming pores. Lysozymes, metabolic products (lactic acid), antibiotics and bacteriocins can also act as a toxic compound for foreign competitors (James *et al*., 1991; Tagg *et al*., 1976; Riley and Wertz, 2002). Many substances play a key role in bacterial interactions and among them bacteriocins are highly specific and efficient antagonists (Sahl, 1994). Bacteriocins are heterogeneous antimicrobial compounds produced by microorganisms and have antibacterial activity against other species. The genes responsible for the production of bacteriocins mostly exist in autosomal state and associated with plasmids (Van Belkum *et al*., 1989).

The genetic information of most of the organisms is encoded in DNA, but few viruses have ribonucleic acid (RNA) as their genetic material. Sometime in addition to DNA and RNA, some extra chromosomal elements are also present in the cytoplasm called plasmids. The plasmid constitutes 1.0 to 2.0% of the total cellular DNA and more than one plasmid may be present in one bacterium. Many non-lanthionine containing bacteriocins are also encoded by plasmid borne genes (Jack *et al*., 1995).

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The biologically active molecule of bacteriocins is formed as a result of post translational modifications of pre-peptides. This modification is due to the production of certain proteins, encoded by gene clusters of bacteriocin as in case of lantibiotics (class I) bacteriocins. While in class II bacteriocins there is no post translational modification for processing and export of biologically active bacteriocin molecule (Deegan *et al*., 2006). With the advancement in genomics and proteomics studies bacteriocinogenic factor can be transferred to the recipient strains through conjugation. Using transduction and transformation methods, specific genes encoding different bacteriocins are harbored in a single strain and can be transfer to non-bacteriocinogenic strains making the incompetent cells to competent level (Reeves, 1972; Dastidar *et al*., 1974). The current study was designed to determine the location of the gene responsible for the production of a novel bacteriocin (BAC-IB17) that was produced by *Bacillus subtilis* KIBGE-IB17.

MATERIALS AND METHODS

Bacterial strain and growth medium

Different strains were isolated and screened for the production of bacteriocin. For the initial screening of both the producer and indicator strains stab and overlay and cross and streak methods were used. Bacteriocin produced by *Bacillus subtilis* KIBGE-IB17 was designated as BAC-IB17 (Ansari *et al*., 2012). Production of BAC-IB17 was enhanced by optimizing various physical and chemical parameters and for maximum production of BAC-IB17 modified TY medium was used (Ansari *et al*., 2012).

Growth kinetics of B. subtilis KIBGE-IB17

Growth kinetics in terms of total bacterial mass of *B. subtilis* KIBGE-IB17 was studied along with the

production of bacteriocin at various stages of microbial growth cycle (Rajaram *et al*., 2010). For this purpose, absorbance of the fermentation broth was determined at 600 nm for different time intervals. Bacteriocin production was monitored at different stages of growth cycle of producer strain in terms of arbitrary units (AU ml^{-1}).

Localization of bacteriocin positive marker (Bac$^+$)

For the determination of location of bacteriocin positive marker, plasmid curing technique was performed. For this purpose, two different intercalating agents including ethidium bromide and acridine orange as well as one anionic surfactant known as sodium dodecyl sulphate (SDS) were used. Bacteriocin producing strain was grown at 37°C for 24 hours. Overnight culture (200.0µL) of the producer strain was then transferred into fresh nutrient broth tube and it was re-incubated at 37°C for just 8.0 hours. This logarithmic phase culture (200.0µL) was further transferred in several nutrient broth tubes (2.0ml) containing ethidium bromide and acridine orange separately in a concentration ranging from 0.025 to 1.0 mg ml^{-1}. SDS was used in different concentrations ranging from 10.0 to 100.0mg ml^{-1} and tubes were incubated at 37°C for 24 hours using producer strain grown without any curing agent as a control. After 24 hours, the tubes with the lowest concentration of the curing agents having growth of the producer strain were selected and further diluted ranging from 10^{-1} to 10^{-4}. To obtain isolated colonies of the culture, a lawn was prepared from each dilution (100.0µL) on nutrient agar plates and these plates were incubated at 37°C for further 24 hours. The isolated colonies obtained were analyzed for the plasmid-mediated bacteriocinogenesis using overlay method. The nutrient agar plates containing isolated colonies of different dilutions were overlaid with 5.0 ml nutrient soft agar containing 0.1 ml of standardized inoculum of the indicator strain that contained 10^8 CFU ml^{-1}. Plate was re-incubated at 37°C for further 24 hours to observe clear zone of inhibition around the producer strains treated for plasmid curing (Hanlin *et al*., 1993; Rasool *et al*., 1996). The colony forming units (CFU) of the indicator strain was standardized using plate count method and McFarland turbidity standard procedure (Smibert and Krieg, 1994).

Isolation of plasmid

For the confirmation of curing technique, the plasmid was isolated from both treated and untreated producer cells. Overnight culture (1.5ml) of bacterium was centrifuged at 35060g for 5.0minutes and the supernatant was discarded. Cell pellet was suspended in 100.0µl of suspension buffer (gL^{-1}: Tris-HCl, 3.8; EDTA, 3.7; glucose, 9.0) and was mixed thoroughly. In this tube 150.0µl of denaturation solution (gL^{-1}: NaOH, 8.0; SDS, 10.0) was than incorporated and mixed gently by inverting several time and kept at 30°C for 5.0 minutes. In the next step 200.0µl of neutralization solution (5.0M potassium acetate; glacial acetic acid, 11.5ml) was added and again kept at 30°C for 10.0 minutes. After incubation, it was centrifuged at 35060g for 5.0minutes and the supernatant was separated in a fresh tube. Absolute ethanol was added up to 2.5 volumes and centrifuged at 35060g for 10.0minutes and the tube was kept at -20C for 20.0minutes. The supernatant was discarded and the pellet was washed with 200.0µl of 70.0% ethanol. The tube was spin for 1.0 minute and the supernatant was again discarded whereas, the pellet containing the DNA was vacuum dried. Sterilized double deionized water (30.0µl) was added to the pellet and the DNA suspension was stored at -20C for further analysis.

Visualization of plasmid on agarose gel electrophoresis

Plasmid was visualized by agarose gel electrophoresis (1.0%). The gel was prepared in 1.0*x* TAE buffer. Ethidium bromide (2.0µl) was incorporated in the gel solution as an inter-chalating agent for trans-illumination. The sample was mixed with the bromophenol blue (6.0 *x*) as a loading dye. Electrophoresis was carried out at 80.0 volts for 45.0 minutes. The extracted plasmids DNA was visualized in gel documentation system under UV trans-illuminator.

RESULTS

Bacillus subtilis KIBGE-IB17 [GenBank: HQ588347] was used in the current study for the production of bacteriocin (BAC-IB17). Production of BAC-IB17 was observed at different stages of the growth cycle of the producer strain and the results revealed that production of BAC-IB17 was time dependent. It was observed that bacterial cell multiplication and bacteriocin production began after the lag period. The production of BAC-IB17 was initiated in the early exponential phase (6.0-18.0 hours) and reached to its maxima at 18.0 hours of incubation (fig. 1). The total cell mass of bacterial culture was also higher at this stage (6.8 Log CFU ml^{-1}) and after 18.0 hours, the bacterial growth started to decline. The arbitrary units of BAC-IB17 remained stable until the late stationary phase (18.0-30.0 hours). However, after 30 hours of incubation, both the cell mass and arbitrary units of bacteriocin showed drastic decline.

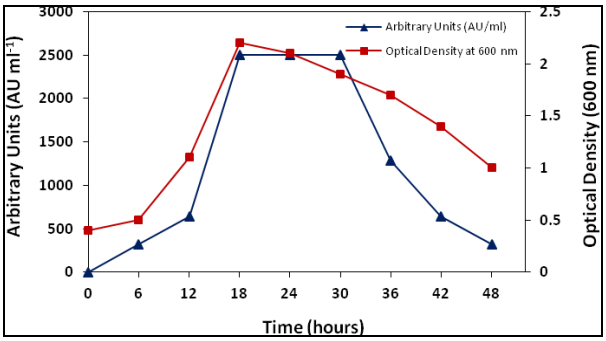

Fig. 1: Growth kinetics of BAC-IB17 along with total cell mass of *Bacillus subtilis* KIBGE IB17 at different stages of growth cycle.

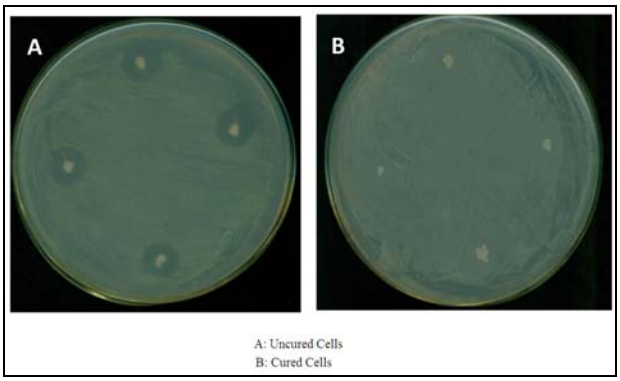

Fig. 2: Overlay method for the confirmation of plasmid curing from *Bacillus subtilis* KIBGE-IB17.

Plasmid curing technique revealed that the gene responsible for the bacteriocinogenecity in *B. subtilis* KIBGE-IB17 was located on the plasmid. Different inter-chelating dyes (ethidium bromide, acridine orange) and sodium dodecyl sulphate were used for the curing of bacterial plasmid. It was observed that only the treatment with ethidium bromide (0.25mg/ml^{-1}) resulted in curing of the plasmid from the producer cell while acridine orange and SDS showed no curing effect. Overlay method was also performed for the confirmation of loss of bacteriocinogenecity after removal of plasmid from the cell (fig. 2). Initially after curing 240 colonies were obtained, out of which 101 colonies showed the zone of inhibition against the indicator strain called uncured colonies, while the colonies that did not showed any inhibitory zone were considered as cured (table 1). About 58.0% of bacterial colonies were cured with ethidium bromide suggesting the possibility of presence of bacteriocin positive marker (Bac^{+}) on its plasmid. Plasmid was isolated from both the cured and uncured colonies in order to confirm the location of the gene responsible for the bacteriocinogenic property of the producer strain. Agarose gel electrophoresis showed that uncured colonies (fig. 3; lane A, C, F and G) contained plasmid as compared to the cured colonies (fig. 3; lane B, D and E) which have lost their plasmid after the treatment with ethidium bromide.

Table 1: Percentage of Cured Colonies for the Location of Genetic Determinant (Bac^{+} Marker) of BAC-IB17

Curing Agent	Total Colonies	Cured Colonies	Uncured Colonies	Curing Percentage (%)
Ethidium Bromide (0.25 mg ml^{-1})	240	139	101	58
Acridine Orange (0.25 mg ml^{-1})	184	0	184	0
SDS (10.0 mg ml^{-1})	196	0	196	0

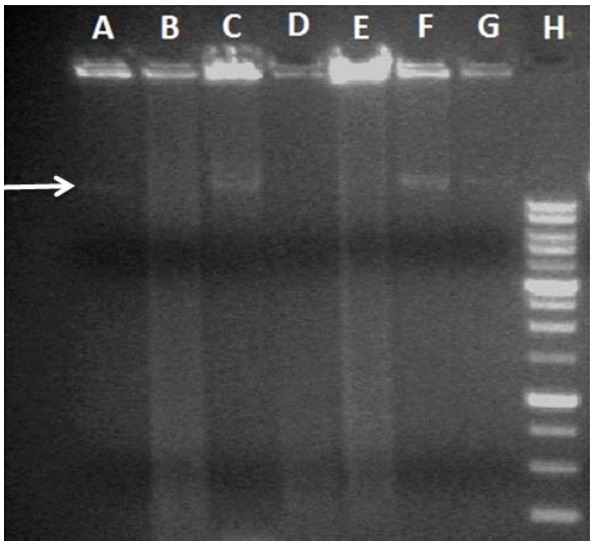

Fig. 3: Agarose gel electrophoresis showing plasmid of cured and uncured cells of *Bacillus subtilis* KIBGE-IB17 using plasmid curing technique.
A: Uncured cells; B: Cured cells; C: Uncured cells; D: Cured cells; E: Cured cells; F: Uncured cells; G: Uncured cells; H; Ladder (1.0 kb). Arrow represents the band of plasmid.

DISCUSSION

Production of any bacteriocin is considered a ubiquitous anti-competitor strategy in bacterial ecosystem. Bacteriocins are heterogeneous and diversified antibacterial proteins in terms of their mode of action, molecular weight, activity spectra, immunity mechanism and target sites. The gene responsible for the production of bacteriocin is mostly located on the bacterial plasmid which also encodes multiple immunity genes. Bacteriocins also play an important part in the maintenance of the plasmid in any bacterial population. Most of the plasmid borne bacteriocins specifically, enteric bacteriocins demonstrates an aspect of bacteriocin evolution and have a typical colicin gene cluster composition (Riley and Wertz, 2002). The advantage of plasmid borne bacteriocins is that due to the transfer of plasmid from one bacterium to another, the non-bacteriocinogenic strains can also become bacteriocinogenic in nature. Hence, the aim of the present study was to localize the gene responsible for bacteriocinogenecity in *B. subtilis* KIBGE-IB17.

Several bacterial strains were previously isolated and were screened for production of broad inhibitory spectrum bacteriocin (Ansari *et al*., 2012). Growth kinetics of *B. subtilis* KIBGE-IB17 along with the production of BAC-IB17 showed that it exhibits secondary metabolite kinetics. The drastic declined in the production of BAC-IB17 resulted in less antibacterial activity after 30 hours of incubation could be either due to the induction of extracellular endogenous proteolytic enzymes during the decline phase or protein aggregation

in the later stages. Most of the producer strains also produce some other proteins which could act as antidotes (immunity proteins) in order to help their survival in the presence of its own antibacterial agent. In the late stage of the cell growth of *B. subtilis* KIBGE-IB17 (decline phase) there might be a possibility that these antidotes became denatured due to long incubation time or their production have ceased which ultimate resulted in the killing of the producer strain. Tarelli *et al*. (1994) and Callewaert and Vuyst (2000) have suggested that these effects might be due to the low pH of the medium after stationary phase or adsorption of bacteriocin on the surface of producer cells resulting in formation of protein aggregates. Similar observations were obtained when *Lactococcus lactis* isolated from marine environment was used for the production of a bacteriocin (Rajaram *et al*., 2010). Alam *et al*. (2011) also reported that a BLIS from *Bacillus subtilis* BS15 was also produced during the late exponential phase or at the beginning of stationary-phase of producer strain. Another bacteriocin from *Bacillus thuringiensis* subsp. *entomicidus* was produced at the mid log-phase and reached its maxima at the early stationary phase (Cherif *et al*., 2008). However, some of the reports on production of bacteriocins demonstrated that it is not necessary that bacteriocins always produced during late logarithmic or early stationary phase of cell cycle. In fact, in some reports the bacteriocin production was observed throughout the experimental growth phase of bacteria (Joerger and Klaenhammer, 1986; Piard *et al*., 1990). It was confirmed from the results that BAC-IB17 was a secondary metabolite therefore it may have several advantages. Although microbial secondary metabolites are not essential for the growth of the bacteria as they produced during secondary metabolic processes (Ruiz *et al*., 2010). In most of the bacteria various groups of enzymes are involved in the production of secondary metabolites and among them synthases are encoded by a cluster of genes often located on chromosome and infrequently on the plasmid of the bacteria (Demain, 1992). However, most of the bacteriocins are encoded by the gene generally located on bacterial plasmid (Jack *et al*., 1995). Current study revealed that BAC-17 was a plasmid borne bacteriocin and it was reported that the isolation of a plasmid DNA from any Gram's positive bacteria is not easy because of the rigidity of the bacterial cell wall present in this group of bacteria (Vriesema *et al*., 1996). However, there are very few bacteriocins that have their genetic determinants located on the chromosome such as pneumocin (Mindich, 1966), bacteriocin 28b (Guasch *et al*., 1995) and two other bacteriocins produced by *Lactobacillus brevis* NM 24 and *Lactobacillus fermentum* NM 332 (Mojgani *et al*., 2009). Ghalfi *et al*. (2009) also demonstrated the plasmid borne bacteriocin from one of the most prevalent lactic acid bacteria known as *Lactobacillus curvatus* CWBI-B28. Some other reports have also clarified the production of plasmid-encoded bacteriocins from other Gram's positive bacteria (Karthikeyan and Santosh, 2009). In the last few years numerous concerns have been raised against increased bacterial resistance towards several effective drugs. Especially in third world countries most of the effective drugs have now turn out to be virtually useless against most of the organisms. The threat posed to general public health can be resolved by the discovery of new antimicrobial compounds having wide range of antimicrobial activity. Researchers are more focusing towards the screening of natural antimicrobial compounds as alternative therapeutic agents. Several experiments also reported the *In vivo* activity of bacteriocins as an antibiotic and its role to control drug resistance (Kirkup, 2006). Current study showed that BAC-IB17 has a potential to be used as a drug against newly emerging drug resistant pathogenic organisms because of its broad spectrum of inhibition.

Keeping the significance value of the plasmid borne bacteriocin in view, it is also essential to understand the evolutionary origin and ecology of bacteriocins. It should not only be focused just on the host cell interaction with the relevant bacterial community but also their role in the maintenance of stable inheritance in terms of anti-pathogenecity should also be studied in more detail.

CONCLUSION

Production of antimicrobial substance is an important factor in microbial ecology. *Bacillus subtilis* KIBGE-IB17 is capable of producing a broad inhibitory spectrum plasmid associated bacteriocin (BAC-IB17). BAC-IB17 is an important secondary metabolite which can be potentially used as an alternative therapeutic agent in pharmaceutical industries to improve human health and nutrition.

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