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Review Article

Significance of Gene Knockout for the Detection of *MurI* Gene in *S.Iniae* for Evaluation of Biochemical Properties Associated With Pathogenicity at Molecular Level

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Abstract

Streptococcus iniae is a gram-positive, sphere-shape and pathogenic bacterium that usually causes infections in fishes as well as human. Glutamate racemase is a key enzyme that is involve in synthesis of peptidoglycan layer of bacteria cell wall. Biochemical properties and nature of this enzyme are highly specific and varies among pathogenic species. The unique features structural, molecular and biochemical properties associated with *MurI* gene as well as functional properties of glutamate racemase in the pathogenicity of *S.iniae* remain unclear. The purpose of this review paper focusing on the methods that used for the evaluation of biochemical properties of glutamate racemase for functioning in bacterial cell wall focusing on *MurI* Gene in *S.iniae* as well knockout of *MurI* gene in *S.iniae* also the discussing the methods used for the determination for the biochemical properties associated with pathogenicity at molecular level. Specific primers are used for the gene knockout plasmid construction region for *MurI* gene of bacterial strain such as *S.iniae*. These are further amplified by the PCR. DNA sequencing is use to clone the DNA fragments. The LB medium is the medium especially use for the *S.iniae* wild type (WT) and Δ*MurI* mutant cultures. Bioscreen machine is use to measure the optical density. The special type of the microscope such as fluorescence microscope is uses for the bacterial viability. TSYE agar plates are used for the measurement of the live bacteria in the form of percentage by plating on. SDS-PAGE is uses to check the purity of expressed proteins determined by the SDS-PAGE. This review helpful for drug discovery and development of new antibiotics by utilizing the *MurI* gene as a target against *S.iniae*.

Keywords: Gene knockout, SDS-PAGE, Glutamate racemase, Pathogenicity, *MurI* gene.

INTRODUCTION

Glutamate racemase is a key enzyme that is involve in biosynthesis of bacterial cell wall. This enzyme participates in catalyzing the biochemical reaction of inter-conversion of glutamate into enantiomeric forms such as D-glutamate and L-Glutamate. This enzyme is found in bacteria that belonging to the lactic acid family that function is to supplying the D-glutamate [1].

Glutamate racemase is composed of two cysteine residues that are involved in monoprotic acid and base mechanism. These two cysteine residues catalyze the inter-conversion of D-glutamate into L-Glutamate. Peptidoglycan layer is composed of mainly two amino sugars such as N- acetylglucoseamine and N-acetlymuramic acid. These two amino sugars maintain the osmolytic balance in the bacterial cell wall. D-glutamate mainly synthesizes the cell wall of bacterial by the formation of peptidoglycan that protects the bacteria against osmolytic lysis[2].

Glutamate racemase in *S.iniae* encoded by a single *MurI* gene. While some other species of *bacillus* family encoded mostly two or more genes. *B.subitilis and B.cereus* have two encoded genes of glutamate racemase. Glutamate racemase is not present in human. So, it can be used in formation of drug particularly for the purpose of antibiotic development[3].

Streptococcus iniae is a pathogen that posses certain characteristics such as gram-positive and sphere shaped. This bacterium functionally target and attack on the human body by producing infections such as sepsis, skin inflammation and cellulitis that damage the body. Seafoods are the main source of *S.iniae* infections. Using of seafood without any precautionary measures lead to increase the infection rate of *S.iniae* and

symptoms appear such as severe inflammation in tissues [4].

Streptococcus iniae was first isolated in dolphin in Amazon freshwater in 1970. It is the most common pathogen found in fish. Infections in fish included skin infections, oedema, meningitis and degenerations of many organs. It has infected many fishes living in freshwater including zebrafish, channel cat fish and rainbow trout and seawater animals also lead to the severe mortality of fishes [5,6].

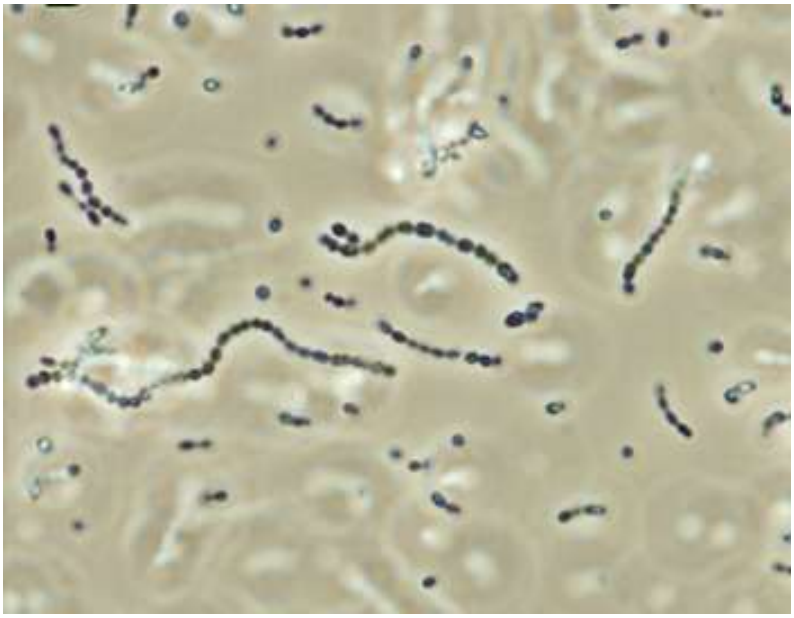

Fig-1: Scanning electron microscope of *S.iniae*

MurI is important gene for the growth of bacterial infections such as *E.coli* [7]. Genes such as RacE and Dat catalyzed the reaction of L-glutamat to G-glutamate in *S. haemolyticus* and *B.sphaericus*. *MurI* gene is not important for them. RacE1 and RacE2 are two genes of glutamate racemase enzymes that are responsible for receamse activity in *B.anhtracis*. Knockout of either RacE1 or RacE2 not affected on bacterial growth in these bacteria [8].

The structural, molecular and biochemical properties of *MurI* Gene in *S.iniae* remain unclear. Functional properties of glutamate racemase in pathogenicity of *S.iniae* not completely described yet. There is need to characterize the different strains of bacteria to understand the unique properties of glutamate racemase for drug discovery and mutational studies for the better characterization of mutants. The purpose of this review paper focusing on the methods that used for the evaluation of biochemical properties of glutamate racemase for functioning in bacterial cell wall focusing on *MurI* Gene in *S.iniae* as well knockout of *MurI* gene in *S.iniae* also the discussing the methods used for the determination for the biochemical properties associated with pathogenicity at molecular level.

Gene Knockout Plasmid Construct Design

Different methods are used for the isolation of strains of bacteria such as *S.iniae.* There are different ways for the gene knock out and plasmid construct design about the *S.iniae*. The laboratory safety rules following according to instructions [9]. The methods involves that mmi1Δ cells generated from a parent strain that containing a deletion of mei4+. This is done by using the minimal medium or EMM Broth supplemented with alenine for 6 days at 30°C. These methods categorized as the gene knockout plasmid construct design, measurement of optical density, assessment of the bacterial viability, whole blood killing assay, expression of *MurI*, purification of *MurI*, measurement of optimum temperature, measurement of thermal stability, measurement of the relative activity, measurement of the optimum pH, measurement of the substrate specificity, measurement of the residual activity of the enzyme [10].

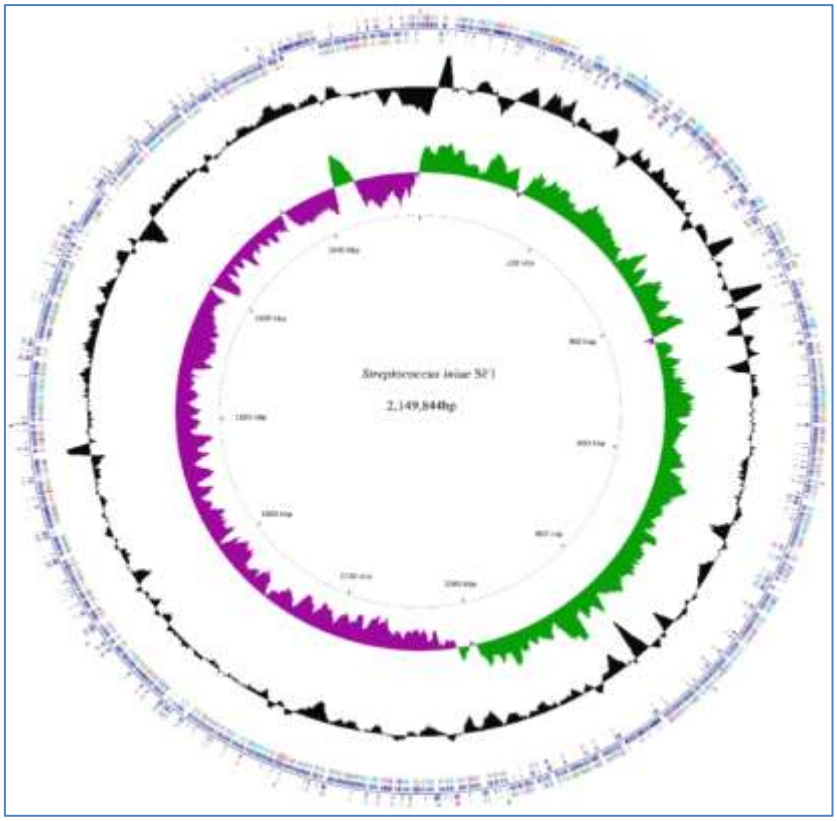

Fig-2: Genome Sequences of the *S.iniae*

There certain steps in designing of the gene knockout construct region for *MurI* gene of *S.iniae* that involves stages of the amplification, cloning gene expression and other biochemical properties of the *MurI* gene[11;12]. Approximately, PCR fragment of 1220 base pairs that containing 620 base pairs locating in the upstream of the ATG of start codon and fragment of the 600 base pairs locating in the downstream of the TCA stop codon of the *MurI* gene undergoes amplification and then next steps is the ligation into the suitable vector[13].

There are different methods to clone the DNA fragments confirmed by advanced molecular techniques. DNA sequencing is used widely to find the correct sequences in the particular species. The first step is to clone the DNA fragments and next step is confirmed find the correct sequences of cloned DNA fragments in the pMZ1. The confirmed pMZ1 plasmid then transformed into the *S. iniae* HNM1 electro-competent cells[14].

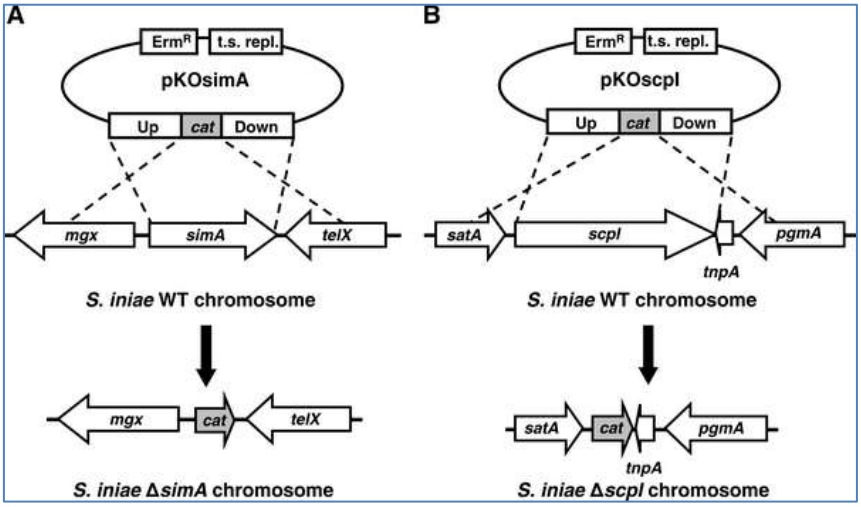

Fig-3: Shows the Gene knock out of *S.iniae* simA and *S.iniae* scpI chromosome

This review also involves the macular methods that are used for the amplification of the specific DNA. So, the methods included that to identify the specific region of the particular type of the strain of the bacteria

and then amplified by using the *S.iniae* through PCR with oligonuclotides and Phusion High-Fidelity DNA PAolymerase (Thermo) used according to the manufacturer's instructions. Then, resection enzymes such as *BamHI and XhoI* are used for further PCR products cloned into the suitable vector [15].

In this review, preparation as well as the designing of the plasmid involves that plasmid generated that encoded the GST-tag to amino terminal or N-terninal of Erh1 by 3C protease cleavage site [2,4]. The cultures that grow is used in 1 L of auto-indcucible terrific broth media that containing antibiotics such as ampicillin (100µg/mL) and chloramphenicol (25µg/mL) for 5hours at 37 °C first and then at 20°C for overnight[12]. In this method, harvested cells are obtained. The cells that are harvest at 4000 rcf for 50 minutes and pellet and resuspend in the presence of the 25 mL lysis buffer. The supernatants that obtained then applied on the GSH-sepharose in the presence of lysis buffer [11,13]. The proteins eluted with elution buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 20 mM GSH, 5 mM β-mercaptoethanol). Eluted protein are used to incubated at 10°C for 24hours by using the GST-3C protease and passing through GSH column to remove the GST-tag as well as the GST tagged 3 C protease. Size exclusion chromatography is used to determine the nature of the unbound proteins subject to the size exclusion chromatography using HiLoad 16/60 Superdex 75 column [16].

Expression of *MurI*

Different methods are used for the expression of the Mur I gene. The methods involves the expression of proteins induced when 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) put into the cells of bacterial culture and incubated for 24 hours at 16°C. Supernatant then taken, purified on a NiNTA column that finally bind the nickel to the beads of agarose through chelation [17].

SDS-PAGE is commonly used to find the purity of expressed proteins that separate the proteins on the basis of their molecular masses with the application of electric field and BCA protein assay that function is to quantify the proteins in a particular sample, respectively.

This test is usually involves the cells of the WT and Δ*MurI* grow into the LB medium with D-glutamate at 25°C. After that, cells then separated by centrifugation and washing and finally sonicated in ice water bath. Concentration of L-glutamate as the product of racemization then measured by using kit assay containing such as L-Glutamate assay. Absorbance for this method measured at 492nm by using the Epoch microplate spectrophotometer[18].

The colonies of *S.iniae* wild type and Δ*MurI* mutants *then calculated and* inculcated into the LB medium and reaction mixture shaken at 150rpm within a temperature range 22-28°C. Liquid portion obtains from cell cultures of Wild type and Δ*MurI* and finally optical density taken by using the Bioscreen machine [6].

Assessment of bacterial viability

Different methods are used to find the bacterial viability that involves the mixing the 5µl dye of the reaction mixture to the suspension of bacteria and incubated in the dark at normal room temperature for period of 12 of minutes. Fluorescence appear during microscopic examination and fluorescence appears by using the advanced fluorescence microscope [9]. For this test in the laboratory, samples of bacterial cultures mix with blood containing heparin from *A.sinensis* and incubated for 90 minutes at 34°C. Phosphate buffer saline uses as a negative control. Live bacteria then taken in the form of percentage by plating on TSYE agar plates [16].

Biochemical Properties of *MurI*

Different methods are used for the measurement of the biochemical properties such as optimum temperature, thermal stability, relative activity, optimum pH, pH stability, substrate specificity and residual activity of the enzyme. The thermometer or optimizer is used optimum temperature of the *MurI* gene at the rate of different rates of temperature such as 5, 10, 15, 20, 25 °C etc. Analyzer is used to measure the thermal stability of the *MurI* with different incubating the enzyme for 1.5 hours at 20, 25, 30 and 35°C[19].

Chemistry analyzer is used to calculate the relative as the ratio between residual enzyme activity at different time intervals by using enzyme assay procedure. Ph meter is used to measure the optimum pH at the optimal temperature in 250 mM Britton–Robinson buffer with a pH of 10 with stability with incubating the enzyme for 1.5 hours at different pH such as 7.0, 8.5, and 10[20].

Enzyme assay is used to find the concentration as well as the substrate specificity in a standard reaction mixture that containing 40 mM TrisHCl buffer with pH 8.5 and different rates of concentrations of the specific substrates such as first substrate is L/Dglutamate, second substrate is L/Daspartic acid, third substrate is L/Dhistidine and last substrate is L/Dalanine[17]. The differential parameter is used for the residual activity of the enzyme at 30°C for 15 minutes according to available of the standard procedure for enzyme assay[16,19].

CONCLUSION

It is difficult to characterize and purify *MurI* gene from different pathogens for better the understanding of their nature and unique properties. *MurI* gene can be utilized as a target against *S.iniae* for the drug discovery and development of new antibiotics.

Characterization of biological functions of *S.iniae* critical in the fields of biosciences. This gene in the field of biochemistry and molecular biology helpful in mutational studies *S.iniae* mutant can be used in vaccine development

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