ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 8 (1): 180-183, 2009
ISSN 1682-296X

Selective *in vitro* Activity of Marine Extract on Genes Encoding Membrane Synthesis of Methicillin Resistance *Staphylococcus aureus*

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Abstract: Resistant strain issues of *Staphylococcus aureus* remain a global challenge and strategic drug discovery programs have been initiated to confront the issue through drug design based on infective target site. Methicillin resistant *Staphylococcus aureus* (MRSA) strains treated with a marine extract, exhibiting potential inhibitory activity through plate and tube assays were screened for activity on selected genes, namely genes encoding for important survival structure of bacteria. Bacterial cytoplasmic membrane is a vital structure and a critical barrier separating inside of cell from the environment. Disruption in membrane integrity will result in leakage of internal contents and followed by cell death. The necessity for bacteria to have membranes makes the membrane a practical target. With this premise, studies on MRSA membrane synthesis genes; *msr*R and *mpr*F genes were conducted via molecular biotechnological approaches. The effect of the resistant gene *mec*A was also investigated. Alteration of nucleotide sequence after treatment was observed only in the *mpr*F gene and was not evidence in nucleotide sequence of *msr*R gene. The selective targeting of *mpr*F gene by the marine extract is an invaluable finding which requires further investigations on the feasibility of the target gene to be utilized in the development of anti-infective agent against MRSA. The research constitutes a scientific advancement in the field of medical treatment of drug resistant bacteria and a forefront study of drugs discovery program focusing drugs target genes.

Key words: *mpr*F gene, *Staphylococcus aureus*, infective target site

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a virulent organism that causes substantial infection related morbidity and mortality in hospitalized patients. MRSA is one of the most common causes of bacterial nosocomial infections, accounting for 40-70% of *S. aureus* infections in intensive care units (Zetola *et al*., 2005). The emergence of high levels of penicillin resistance followed by the development and spread of strain resistant to the semi synthetic penicillin (methicillin, nafcillin and oxacilin), macrolides, tetracyclines and aminoglycoside has made therapy of staphylococcal diseases a global challenge. The rate of MRSA infection is increasing and treatment is not only expensive but available drug of choice is toxic to human host. These study concentrate on the bacterial target site complement to antibacterial mechanisms would improve the efficacy and prevent the emergence of microbial resistance can be good alternative than taking hazardous drugs (Maillard, 2002).

To combat MRSA phenomenon, massive productions of antibacterial compound in pharmaceutical industry are regulated, but this new generation of antibiotics must be accompanied by an identification of bacterial target with therapeutic values. The present study was conducted with aim to screen for potential genes as infective target and genes involved in membrane synthesis are hypothesised to be suitable target for drug or developing the target as therapeutic molecule. Two genes closely related to membrane production in MRSA, namely *msr*R (Rossi *et al*., 2003), membrane associated protein and *mpr*F (Peschel *et al*., 2004), gene coding for transport protein lysine to phospholipids layer in Methicillin-resistant *Staphylococcus aureus* (MRSA) membrane were investigated besides the *mec*A gene, responsible for the resistance of strain to beta-lactams antibiotics.

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MATERIALS AND METHODS

Sea cucumber extract preparation: Research conducted in Microbiology Laboratory, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia in 2008. The animal used in this study, *Stichopus badionotus* was collected from West Coast, Port Dickson Malaysia. The animals were cleaned thoroughly, oven dried and powdered. The powder (300 g) was soaked with 1 L of methanol and stirred for 48 h. After filtration of the extract, it was evaporated at 50ºC until dried. The dried material was used for further test.

Antibacterial activity: The antibacterial activity of the extract was evaluated against MRSA through Kirby Bauer disc diffusion assay (Bauer *et al*., 1966) and Minimal Inhibitory Concentration (MIC) of extract was determined. A serial dilution of 4/5 dilution factor of extract was made on Mueller Hinton broth. Each tube was inoculated with 100 µL of 0.5 McFarland of the test bacterial and incubated for 6-8 h then streak on Mueller Hinton plates. The MIC was determined after overnight incubation of the plates at 37ºC taken the lowest concentration of extract used to inhibit bacterial growth as MIC.

Molecular assay: Activities of the marine extract on MRSA were performed for prediction of genes affected by the extract. Primers for RT-PCR were design manually based on regions of interest in genes encoding for synthesis of a selected bacterial structure important in survival. The target gene sequences were obtained from the public domain of MRSA database. Genes targeted are genes related to membrane development of MRSA and beta-lactams resistant gene. Primers used sequenced as shown in Table 1. Partial expression profiles of bacterial target genes after treatment with extract and controls were determined using RT-PCR analysis. Methods and reagents used were according to manufacturer's instructions of commercial kit (Master Pure™ Complete RNA extraction kit, Alleights) with slight modifications. Total RNA were extracted from treated and untreated MRSA on MIC's plate using RNA Extraction kit. The total RNA then converted to cDNA using Monster Script™ cDNA kit (Alleights) according to manufacturer's instruction for first strand and second strands before projected to Reverse Transcriptase PCR (RT-PCR) using prior designed primers. PCR were carried out in a total volume of 25 µL based on methods outlined by Oku *et al.* (2004) with slight modifications. The RT-PCR product was run on 1.2% agarose gels and stain in 0.2 µg mL^{-1} of ethidium bromide and viewed under AlphaImager™ 2200 (Alpha Innotech). The bands of interest were extracted with QIAquick gel extraction kit (Qiagen) and sent for commercial sequencing.

RESULTS AND DISCUSSION

Figure 1 shows representative data of the PCR assay for ATCC 29247 and ATCC 700698 strains. The amplified DNA fragment of the *msr*R, *mpr*F and *mec*A, the predicted size of which were 406, 1241 and 533 bp, respectively, could be detected in several strains. No DNA amplification was seen in negative template and method controls. The analysis based on Gene bank database of selected genes encoding for proteins related to membrane development in *Staphylococcus aureus* namely msrR, membrane lysine transport, mprF genes and mecA resistant gene concurred with the published sequence with high similarity values upon amplification with the designed primers. Manually designed primer sets for msrR, mprF and mecA amplified PCR products of sizes 406, 1241 and 533 bp, respectively. The custom

Table 1: List of primers, target genes in *Staphylococcus aureus*

Gene	Source	Sense primer (5'-3')	Antisense primer (5'-3')
mprF	*S. aureus*	GTATCGGGAGTTATCTGG	TCAACCTACGTGCTCTAC
msrR	*S. aureus*	GGTGATAGTCTTCGGCTTTG	GGAGGTTGCTTTTGGTGTA
mecA	*S. aureus*	AAAATCGATGGTAAAGGTTGGC	AGTTCTGCAGTACCGGATTTGC

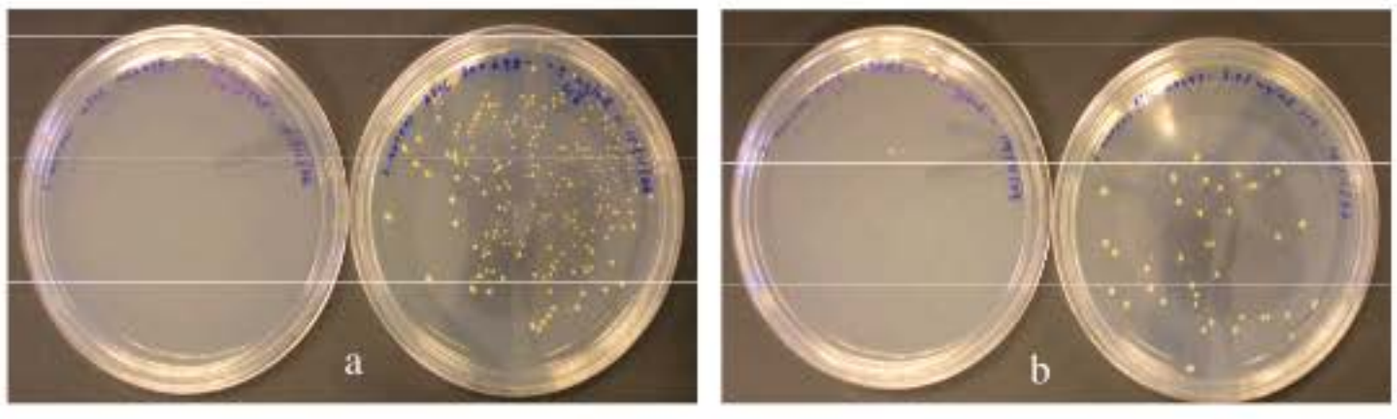

Fig. 1: Plate of Minimal inhibitory concentration of MRSA treated with marine organism extract; (a) ATCC 700698, (b) ATCC 29247

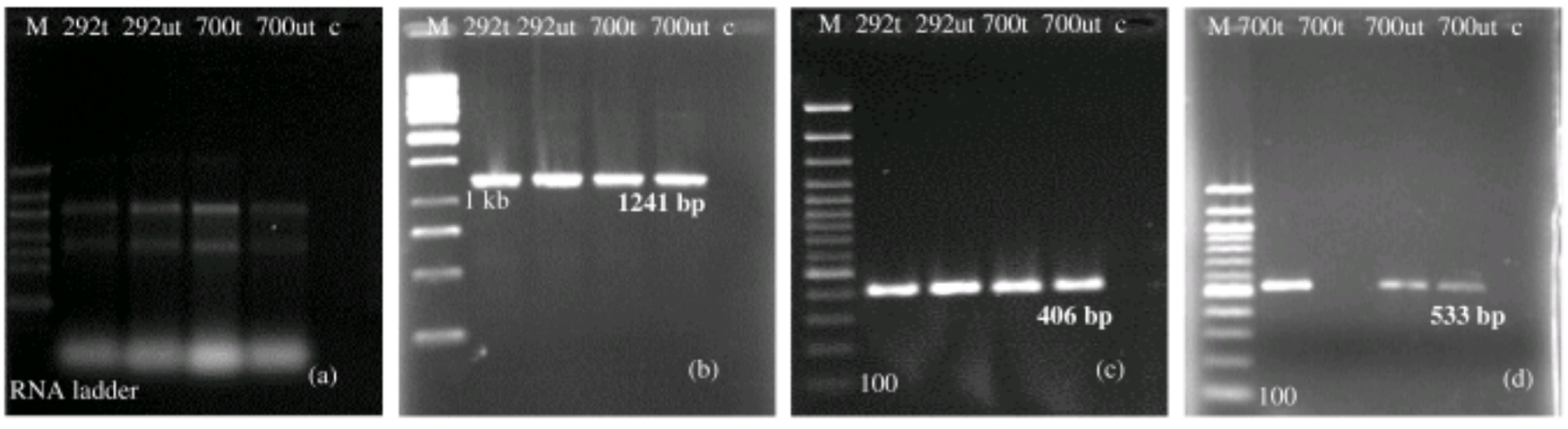

Fig. 2: (a) RNA band of treated and untreated MRSA and non MRSA reference strain, ATCC 700698 and ATCC 29247. (b) Amplification of a band from extracted RNAs at a size of 1241 bp. (c) Amplification of a band from extracted RNAs at a size of 406 bp and (d) Amplification of a band from extracted RNAs at a size of 533 bp

(a)

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29247U_membF_complete          TGGCGACATTCTTCACTTACGCTTCATATATTTTAATAACATGGTTAGCT
membF_[921_-_2180]_AF145699    TGGCGACATTCTTCACTTACGCTTCATATATTTTAATAACATGGTTAGCT
29247T_mprF_complete           TGGCGACATTCTTCACTTACGCTTCATATATTTTAATAACATGGTTAGCT
                               ***************************************************

29247U_membF_complete          ATTATTTTTGTTCTGCTTATTGTAGCTTTCCGTAGAGCACGTAGGTTGA-
membF_[921_-_2180]_AF145699    ATTATTTTTGTTCTGCTTATTGTAGCTTTCCGTAGAGCACGTAGGTTGAA
29247T_membF_complete          ATTATTTTGTGGCGACATTCTTCACTTACGCTTCATATATTTTAAT-AAC
                               ********    *  * *  *  *  *   * *     *  *   *  *
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(b)

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700698t_msrR_complete          TTCTTTTTGATGATCATTAGTGTAGAAAATAGCTGTTTGATATTGAGGAC
msrR_[gene_bank]               TTCTTTTTGATGATCATTAGTGTAGAAAATAGCTGTTTGATATTGAGGAC
700698U_msrR_complete          TTCTTTTTGATGATCATTAGTGTAGAAAATAGCTGTTTGATATTGAGGAC
                               ***************************************************

700698t_msrR_complete          CACGGTCTTGATATTGACCACCTGTATCTAATGGGTCAATGACTGAGAAA
msrR_[gene_bank]               CACGGTCTTGATATTGACCACCTGTATCTAATGGGTCAATGACTGAGAAA
700698U_msrR_complete          CACGGTCTTGATATTGACCACCTGTATCTAATGGGTCAATGACTGAGAAA
                               ***************************************************
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(c)

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700_untreated_sc6_complete     TTGATGCAATTGAAGATAAAAATTTCAAACAAGTTTATAAAGATAGCAGT
Mec_a_gene_bank                TTGATGCAATTGAAGATAAAAATTTCAAACAAGTTTATAAAGATAGCAGT
700_treated_sc6_complete       TTGATGCAATTGAAGATAAAAATTTCAAACAAGTTTATAACTTGACCAGT
                               ****************************************    * ****

700_untreated_sc6_complete     TATATTTCTAAAAGCGATAATGGTGAAGTAGAAATGACTGAACGTCCGAT
Mec_a_gene_bank                TATATTTCTAAAAGCGATAATGGTGAAGTAGAAATGACTGAACGTCCGAT
700_treated_sc6_complete       GGTATTTAAAAAAGCGATAATGGTGAAGTAGAAATGACTGAACGTCCGAT
                                 *****  ****************************************
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Fig. 3: Alignment of sequencing results from treated, untreated *S. aureus* and gene bank sequence of a putative membrane synthesis gene. (a) *mpr*F gene (b) *msr*R gene and (c) *mec*A gene

designed primers based on published sequences and the parameters in optimization attempts for PCR produced products in accordance to results illustrated in earlier studies.

A single amplification band of sizes 406, 1241 and 533 bp, for each gene were obtained through RT-PCR analysis. Both strains, ATCC 29247 and 700698 treated with marine extract and untreated as negative controls showed expected amplifications (Fig. 2). The bands upon purification and commercial sequencing however, showed a selective activity of the methanolic extract. In the case of *mpr*F and *mec*A genes, changes in the nucleotide sequences of treated bacterial strains were seen but no changes in nucleotide sequence of *msr*R gene. Untreated control strains exhibited no changes in the genes sequences (Fig. 3a-c). Nucleotide changes in *mpr*F and *mec*A genes after extract treatments indicated successful complementary effect of the extract and the target sites. The concept of membrane acting antibiotics should be further investigated to determine whether this molecules can gave same reaction on Gram negative bacteria by targeting the outer or inner membrane, against which there is also urgent need for novel molecules (Bambeke *et al*., 2007).

In the search for novel antibacterial with anticipation to the identification and exploitation of novel targets, a profound finding is revealed in the present study. The marine extract with ability to inhibit growth of MRSA is shown to affect two of the three genes analyzed. The effect on *mpr*F gene is desirable since it is one of the genes for bacterial membrane synthesis and the effect on *mec*A gives added value of the extract (Staubitz *et al*., 2004; Ruzin *et al*., 2003). Silver (2007) stated that targets should be selected on the basis of their essentiality and presence in the desired spectrum of bacteria, lack of human homologues and theoretical drug ability and encoding for structures that are synthesized by multiple genes. In addition, it was also proposed that multiple targets from monotherapeutic agents could be successful in avoiding build up of resistance. Multiple targets encoding an essential bacterial structure is desirable since the chances of mutation or resistance could be hazardous to the bacteria. The findings from the present study will be a firm basis for future research in developing new antibacterial agent through current approaches of *in silico* biology and combinatorial chemistry in designing inhibitors of unexploited multiple targets and combinations of single-target inhibitors to overcome resistance.

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