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Detection the Antibacterial Effect of Seaweeds on *Staphylococcus aureus* DNA Repair Gene (*adaB*) and Cell Wall Protein Synthesis (*sav1017*) by Molecular Approaches

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Abstract: Polymerase Chain reaction amplification of DNA was performed to used to study the presence and effect of treated and untreated *Stapylococcus aureus* genes *sav1017* and *adaB* with marine seaweeds *Gracilaria changii* and *Euchema denticulatum*. From the sequencing analysis, the changes were detected in the gene sequence of *adaB* and *sav1017*, genes after treated with either *G. changii* or *E. denticulatum* extract, which involved the substitution of the nucleotide base pair and insertion or deletion of the purine or pyrimidine base. The novel of this study is the extract of *G. changii* and *E. denticulatum* interrupting the important function in MRSA and non-MRSA isolates so that this pathogen cannot survive longer than usual. This significant finding can be applied to a medical treatment whereby both of these extracts can be used as an alternative treatment for the infection of *S. aureus* especially to overcome drug resistance treatment problems in MRSA strains.

Key words: Polymerase chain reaction, *Staphylococcus aureus*, seaweeds, antimicrobial activities, extraction, Malaysia

INTRODUCTION

Seaweeds or algae are a eukaryotic organism (Michael *et al*., 2005) that lives in salty water in the ocean and is recognized as a potential source of bioactive natural products. They contain compounds ranging from sterols, terpenoids to brominated phenolic, which show bioactivity against microorganisms (Wong *et al*., 1994). In order to search for new antimicrobial agents, the antibacterial mechanisms of either *G. changii* or *E. denticulatum* extracts on selected genes involved in the metabolism for structural development of the bacteria can be utilized to predict the crucial anti infective target on the pathogen. In this study, *adaB* gene and *sav1017* gene in *Staphylococcus aureus* isolates were chosen to study the activity of *G. changii* and *E. denticulatum* extracts on both genes. The *adaB* gene and *sav1017* gene are the protein coding genes where're *adaB* gene is involved in the synthesis of probable methylated DNA protein cysteine methyltransferase, which is part of the DNA repair system of *S. aureus* cell. The *sav1017* gene is involved in the synthesis of UDP-N-acetylglucosamine or LPS N-acetylglucosamine transferase, which acts in the cell wall or membrane biogenesis. The protein synthesized by *sav1017* gene is similar to cell wall synthesis protein. Since, no previous study or literature were found for both genes, the information for both genes can be found under accession number NC_002758 with gene ID is 1125262 and locus tag is SA2335 for *adaB* gene and gene ID is 1120992 and locus tag is SAV1017 for *sav1017* gene through NCBI website at http://www.ncbi.nlm.nih.gov. In this study, *ogt* gene in *E. coli* was chosen as a negative control to

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compare the effect of *G. changii* or *E. denticulatum* extracts on DNA repair gene in *S. aureus* to the DNA repair gene in *E. coli*. *ogt* gene in *E. coli* is a protein coding gene, which function in the synthesizing of enzyme known as cysteine-protein methyltransferase (O-6-alkylguanine-DNA), which involved in the DNA repair function in the cytoplasm of *E. coli*. Polymerase Chain reaction assay or shortly known as PCR is an *in vitro* process that selectively targets a short segment of DNA sequence and copies it into a million fold or more. It is a technology using molecular biology discovered in 1983 by Kary Mullis (Saiki *et al*., 1985; Rabinow, 1997). This method allows the amplification *in vitro* of a specific region of DNA, in order to produce enough DNA to be adequately tested. A basic PCR set up requires several components and reagents (Joseph and David, 2001). The tremendous sensitivity of PCR allows target genes to be detected when present in extremely low concentrations, thereby permitting the detection of a minor bacterial sub population within a complex mixed flora without the need for isolation of the organisms of interest (Lo, 1994; Johnson and Brown, 1998).

MATERIALS AND METHODS

Propagation of untreated bacterial culture: A single pure colony of *S. aureus* and *Escherichia coli* isolates was inoculated to a 250 mL capacity Enlermeyer flask containing an adequate volume of sterilized *Luria bertani* broth (Invitrogen Inc.). The broth was then incubated overnight at 37°C with constant shaking on the shaker. This was followed by the procedure of the DNA extraction using the GeniSpin™ Bacterial DNA Kit (BST^{Techlab}).

Propagation of bacterial culture treated with *Gracilaria changii* and *Euchema denticulatum* extract: A single pure colony of *S. aureus* isolates was inoculated to a 250 mL capacity Enlermeyer flask containing an adequate volume of sterilized *Luria bertani* broth (Invitrogen Inc.) containing either 50 mg mL^{-1} *G. changii* or 40 mg mL^{-1} *E. denticulatum* extract. The broth was then incubated 4-5 h at 37°C with constant shaking on the shaker. This was followed by the procedure of the DNA extraction using the GeniSpin™ bacterial DNA kit (BST^{Techlab}).

DNA extraction: Five isolates of *S. aureus* including MRSA and non-MRSA strains were extracted using the GeniSpin™ Bacterial DNA Kit (BST^{Techlab}). One *E. coli* strain, which serve as negative control was extracted by using the same method. The culture was aliquot into 1.5 mL microcentrifuged tube and pelleted until an adequate quantity of bacteria was obtained. Cells then were resuspended in 100 µL TE buffer and the bacterial cell wall was removed by lysozyme (10 mg mL^{-1}) digestion and followed by buffer BTL and proteinase K (15 mg mL^{-1}) digestion. RNaseA (25 mg mL^{-1}) was added to remove the RNA, which normally co-purifies with genomic DNA. Following lysis, binding condition was adjusted and the sample was applied to an I-Spin™ column after adding the Buffer BDL and absolute ethanol.

Two rapid wash steps using wash Buffer will removed trace salt and protein contaminants and finally DNA was eluted in water or low ionic strength buffer. This DNA can be directly used in downstream applications without the need for further purification. The eluted DNA was then run onto 0.8% agarose gel electrophoresis to check the present of genomic DNA and stained with ethidium bromide to visualize under transilluminator. Lastly, the DNA was stored at -20°C in a refrigerator.

Polymerase Chain Reaction (PCR): PCR amplification of DNA sequences was performed to confirm the presence of *sav1017*, *adaB* in *S. aureus* and *ogt* genes in *E. coli*, by using the corresponding primers shown in Table 1. All the primers that were utilized in this study were synthesized by Research Biolabs, Singapore. The sequences of primers for the amplification of *sav1017*, *adaB* and *ogt* gene were designed using computer-assisted software (Hitachi). Using the Thermal Block Cycler (T-Personal) provided by Biometra, amplification of *sav1017*, *adaB* and *ogt* gene were achieved according to data represented in Table 2 and 3.

Table 1: The sequences of primers used in detection and amplification of genes

Gene	Sequences	Size (bp)
sav1017	5'-CTTGACCAGGTGCAGGAT-3'	
	5'-TATTACAGCCGCCCAGA-3'	691
adaB	5'-CAACCGCTTGGGCTGACA-3'	
	5'-CGCCTGTAGGACGATTGG-3'	321
ogt	5'-TGCGGGCGGTTGAATGG-3'	
	5'-CGACGATGCTGATGGGATT-3'	333

Table 2: The reaction mixture for each primer

Primer	*sav101*	*adaB*	*mecA*	*mecR1*	*mecI*	*ogt*
	(µL)					
MgCl2	0.3	0.3	0.4	0.4	0.4	0.4
dNTP	0.4	0.4	0.4	0.4	0.4	0.4
PCR buffer	2.5	2.5	2.5	2.5	2.5	2.5
Forward primer	0.1	0.1	0.1	0.1	0.1	0.1
Reverse primer	0.1	0.1	0.1	0.1	0.1	0.1
*Taq*DNA olymerase	0.2	0.2	0.2	0.2	0.2	0.2
Genomic	1.0	1.0	2.0	2.0	2.0	2.0

Table 3: Thermal cycling profile for each primer

Primer	Initial denaturation	Extension denaturation	Annealing temperature	Elongation	Final extension	Cycle
sav1017	96°C (5 min)	95°C (1 sec)	59°C (30 sec)	55°C (30 sec)	55°C (30 sec)	35
adaB	96°C (5 min)	95°C (1 sec)	59°C (30 sec)	55°C (30 sec)	55°C (30 sec)	35
ogt	96°C (2 min)	94°C (1 min)	58°C (1 min)	72°C (1 min)	72°C (7 min)	40

RESULTS AND DISCUSSION

Amplification of various genes through Polymerase Chain Reaction (PCR) with respective specific primers (Table 1) were sensitive at the DNA template concentration of 100 ng μL^{-1} for *S. aureus* and *Escherichia coli* genomic DNA (Fig. 1 and 2). This study demonstrates that the *adaB* 321bp (Fig. 3) and *sav1017* 691 bp (Fig. 4) genes were successfully amplified in 5 isolates of the *ogt* gene which served as a control gene was also successfully amplified from one *E. coli* isolate (Fig. 5) with a size of 333 bp in untreated and treated *E. coli* isolates. All the isolates treated with either *G. changii* or *E. denticulatum* extracts also showed the amplification of these genes whereby a single band corresponding to the respective PCR products were observed.

Fig. 1: The genomic DNA extracted from untreated and treated *Staphylococcus aureus*. Lanes 1-5: The non-treated isolates. Lanes 6-10: The isolates treated with *Gracilaria changii*. Lanes 11-15: The isolates treated with *Euchema denticulatum* extract. Lane M: The Lambda

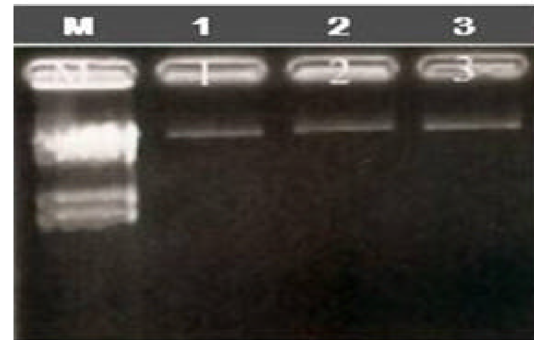

Fig. 2: The genomic DNA extracted from untreated and treated *Escherichia coli*. Lanes 1: The untreated isolate. Lane 2: The isolate with *Gracilaria changii*. Lane 3: The isolates treated with *Euchema denticulatum* extract. Lane M: The Lambda hind 3 molecular weight marker

Sequencing analysis: Sequencing analysis of amplified products with published gene sequences. Since, the objective of sequence analysis study is to compare between the sequences of the untreated genes with the sequences of the genes treated with either *G. changii* or *E. dentiulatum* extracts, the software in the Biology workbench 3.2 under alignment program (http://workbench.sdsc.edu) were used and results clearly illustrated degrees of similarities and differences between untreated isolates, treated isolates and the sequence from the Gene Bank database. Upon aligning of each gene, several mutations, which include changes, deletion or insertion of nucleotides were noted after the alignment using ClustalW program.

The changes or mutation in nucleotides of the genes detected is correlated to the effect of the seaweed extracts and being assumed to predict inhibitory mechanisms of the extract. Table 4 and 5 showed the type and number of nucleotide changes found in the sequences of the gene's sequences treated either with *G. changii* or *E. denticulatum* extract. According to the results, only the sequences *ogt* gene do not show any change after treatment with either *G. changii* or *E. denticulatum* extract. *adaB* gene sequences showed obvious changes. In this study, the pattern of nucleotide changes for *adaB* and *sav1017* treated with *G. changii* extracts is similar to the pattern of nucleotide changes for both genes treated with *E. denticulatum* extracts.

The *adaB* is the gene encoding protein, which is involved in the synthesis of methylated DNA-protein cysteine methyltransferase. The enzyme produced by this gene plays an important role in the DNA repair of *S. aureus* cell. In the present study, the results obtained through molecular analysis, could probably predict the possible antimicrobial mechanisms caused by either *G. changii* or *E. denticulatum* extract on *S. aureus* through the effect of extracts on the sequences of *adaB* gene. Through sequencing analysis by using the ClustalW program, the findings underlined that inhibition effect of both seaweeds extracts may be chromosome mediated evidenced by the changes of chromosome encoded genes. In *adaB* gene, the changes in nucleotides evidenced from the gene sequence treated with either

Fig. 3: Amplification of *adaB* gene by PCR in untreated and treated *Staphylococcus aureus*. Lane M: The 100 bp molecular weight marker (MBI fermentas). *adaB* gene positive isolates have a single band present in the region between the ladders of 300-400 bp. The actual band position is at 321 bp. Lane C: A negative control showing no band indicating that there was no contamination during the process

Fig. 4: Amplification of *sav1071* gene by PCR in untreated and treated *Staphylococcus aureus*. Lane M: The 100 bp molecular weight marker (MBI fermentas). *sav1071* gene positive isolates have a single band present in the region between the ladders of 600-700 bp. The actual band position is at 691 bp. Lane C: A negative control showing no band indicating that there was no contamination during the process

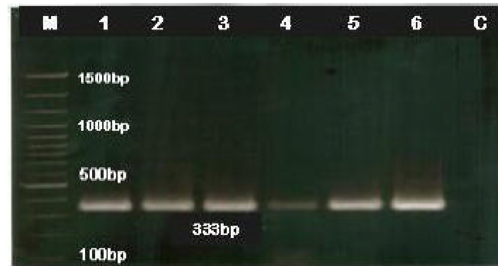

Fig. 5: Amplification of *ogt* gene PCR. Lane 1: The band of the untreated genomic. Lane 2: The gene of genomic treated with *G. changii*. Lane 3: The gene of genomic treated with *E. denticulatum*. Lane 4: The band of gene of untreated cDNA. Lane 5: The gene of cDNA treated with *G. changii*. Lane 3: The gene of cDNA treated with *E. denticulatum*. Lane M: The 100 bp molecular weight marker. *ogt* gene positive isolates have a single band present in the region between the ladders of 300-400 bp. The actual band position is at 333 bp. Lane C: A negative control showing no band indicating that there was no contamination during the process

G. changii or *E. denticulatum* extracts, may affect the functional property of that gene. The changes in nucleotides base pairs of *adaB* gene sequence were in the form of point mutation which the changes involved either a substitution of nucleic acid base pair, insertion of a new acid nucleic base pair into the amplified *adaB* gene sequence or the deletions of base pair from the amplified *adaB* gene sequence. A substitution is a type of mutation that causes the replacement of a single base nucleotide with another nucleotide. This type of mutation can be assumed as a silent mutation whereby the mutation does not result in a change to the amino acid sequence of a protein. Normally this mutation may occur in a non-coding region (outside of a gene or within an intron). Therefore, we can assume that the replacement of a single base nucleotide with another nucleotide within *adaB* gene sequences may not alter the function of this gene. However, the insertions or deletions of a single base pair into or from *adaB* gene sequences could possibly change the function of this gene since this type of mutation have more of an adverse effect on the synthesized protein due to nucleotides still being read in triplets, but in different frames. This type of mutation was called as a frame shift mutation. In this study, we can predict that the frame shift mutation through insertion or deletion of nucleotide base pairs may affect the DNA yielded during the transcription and translation p rocess, which results in alteration in the function of methyltransferase enzyme in *S. aureus* isolates.

Methyltransferase enzyme is an enzyme that acts by transfer the methyl group in *S. aureus* cell from a protein involved in the DNA repair of the *S. aureus* isolate, the

Table 4: Type and number of nucleotide changes found in the sequences of the genes after *S. aureus* treated with *G.changii* extract

				Genomic			cDNA		
Gene	ID	Changes in genomic (%)	Changes in cDNA (%)	Changes	Insertion	Deletion	Changes	Insertion	Deletion
adaB	20	4.30	0.0	7	5	-	-	-	-
	N8	1.40	1.2	2	1	1	1	1	-
	ST5	2.90	1.7	2	2	1	5	-	-
	ST8	6.20	9.8	8	3	6	25	2	2
	ST9	1.80	0.0	3	-	2	-	-	-
sav 1017	20	1.30	0.0	9	-	-	-	-	-
	N8	1.20	0.0	5	2	1	-	-	-
	ST5	13.20	0.0	62	22	-	-	-	-
	ST8	0.40	0.0	4	-	-	-	-	-
	ST9	5.90	0.0	30	9	-	-	-	-

Table 5: Type and number of nucleotide changes found in the sequences of the genes after *S. aureus* treated with *E. denticulatum* extract

				Genomic			cDNA		
Gene	ID	Changes in genomic (%)	Changes in cDNA (%)	Changes	Insertion	Deletion	Changes	Insertion	Deletion
adaB	20	1.10	0.3	2	-	1	1	-	-
	N8	1.10	0.3	2	-	1	1	-	1
	ST5	1.30	1.7	1	-	11	4	-	1
	ST8	2.20	2.1	6	-	-	4	-	2
	ST9	0.00	0.0	-	-	-	-	-	-
sav 1017	20	14.70	0.0	79	16	4	-	-	-
	N8	0.20	0.0	1	-	6	-	-	-
	ST5	5.20	0.5	27	6	-	3	-	-
	ST8	0.60	0.0	4	-	-	-	-	-
	ST9	0.50	0.0	-	3	1	-	-	-

cysteine. Due to the environmental stressors such as the exposure to chemical, inappropriate temperature and culture medium, the methyl group from the environment will get attach to a protein involved in the DNA repair of the *S. aureus* isolate, the cysteine. The combination of methyl group with cysteine will effectuate with the DNA repair process. In this case, the *adaB* gene will produce the methyltransferase enzyme whereby this enzyme then will activate the methyltransferase activity to remove the methyl group from the cysteine.

Therefore, the broken DNA in *S. aureus* will be repaired and *S. aureus* will survive as long as possible. In contrast, the addition of either the methanol extract of *G. changii* and *E. denticulatum* into the *S. aureus* culture at the early of the experiment may disrupt the normal function of *adaB* gene in producing the methyltransferase enzyme. This is because the changes in *adaB* gene sequences, which involved a frame shift mutations through insertion or deletion of nucleotide base pairs in this gene's sequence may change the original DNA and RNA function of this gene. According to Michael *et al.* (2005), the changes in DNA and RNA will produce the new protein through the translation process. It is because when RNA is changing, the translation process will produce different proteins. Therefore, the original gene will not be expressed. So that in this case, the affected *adaB* gene failed to express the same protein as in the untreated *S. aureus* culture. As a consequence, events that entail could be methyltransferase enzyme with altered function or no methyltransferase enzyme yielding through translation process. Without functional methyltransferase enzyme, the DNA repair function in *S. aureus* cannot take place whereby the methyl group failed to be expelled from the cysteine. In other words, often DNA damage itself can induce DNA repair systems but in this case, the *S. aureus* cannot survive so it will die or reduce in growth. Normally DNA repair system do not make mistakes, however, when this extract affect the DNA repair gene, DNA repair process seem to be error-prone and it is the repair processes itself that introduces the mutation.

The *sav1017* gene is correlated with the cell wall synthesis in *Staphylococcus aureus* since the gene is involved in the synthesis of enzyme known as UDP-N-acetylglucosamine tranferase which acts in the cell wall biogenesis and consequently could provide an attractive target for the design of antibacterial agents. Therefore, based on the changes of the treated nucleotide sequence in *sav1017* gene, the seaweeds extracts discussed in the current study are predicted to interfere with specific events that are essential for bacterial growth, more specifically, cell wall biosynthesis. In this study, the *S. aureus* cell wall biosynthetic pathway will be discussed, so that the predicted inhibiting mechanism of either *G. changii* or *E. denticulatum* extracts and their mode of action on *sav1017* gene can be more easily

understood. Cell wall is a rigid structure outside the cytoplasmic membrane which provides support and protection from osmotic lysis. It also gives shape and rigidity to the cell.

Most prokaryotes such as bacteria cannot survive without their cell walls (Michael *et al*., 2005). The function of *sav1017* gene might be assumed to perform peptidoglycan synthesis. Supplement of either methanol extract of *G. changii* or *E. denticulatum* to the *S. aureus* culture medium in the current study have shown changing the sequence of the nucleotides for this gene either by changes in nucleotide base pair or insertion and deletion of nucleotide base pair after amplification by polymerase chain reaction assay, which can lead to phenotypic change. According to Michael *et al*. (2005), a single base-pair insertion or deletion in the sequence of a gene could lead to a dramatic change in the ability of the gene to function because of the increased or decreased expression or a change in regulation. Insertion in the coding region of a gene may alter splicing of the mRNA or cause a shift in the reading frame shift both of which can significantly alter the gene product.

In addition, deletion removes one or more nucleotides from the DNA. Like insertions, these mutations can alter the reading frame of the gene. Therefore, when the seaweed extracts have altered the base of purines or pyrimidines in DNA sequences through frameshift mutations, the original genetic information will change where're the DNA replication will duplicates the altered master genetic blueprint. The altered genetic blueprint in DNA then will be used as template for the transcription process to yield the RNA. The function of the *sav1017* gene in the treated *S. aureus* in this study might be disrupted. Therefore, the peptidoglycan synthesis will not occur and rather than discontinued. After several times, the *S. aureus* in the treated culture will loose the structural integrity and without the proper cell wall, *S. aureus* cannot retain the osmotic pressure from outside and finally the cell will experience lysis whereby water will enter the cells, cell swells and eventually burst (Michael *et al*., 2005).

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