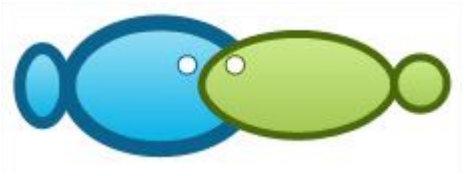

Shotgun sequencing analysis of a metagenome library derived fosmid clone from bacterial community associated with toxic dinoflagellate *Alexandrium tamiyavanichii*

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Abstract. Cultures of dinoflagellates, one of the most important harmful algae group, often contain a considerable population of bacteria probably carried over during isolation of cells for culture. At present, the precise nature of the association of bacteria with dinoflagellates in culture is still remained unclear. Lately, metagenomics has been widely used to elucidate the microbial diversity and functions in nature environment as well as a tool to discover new genes and new natural products. In this study, we attempt to fully sequence and analyse a metagenome library-derived fosmid clone previously constructed from bacterial community associated with toxic dinoflagellate *Alexandrium tamiyavanichii*, in order to provide insights into the potential functional role of the dinoflagellate-associated bacteria at the genomic level. The DNA insert contains ~32.9 Kbp in size with an estimated G+C content of 59.57%. Twenty-six ORFs were predicted of which 7 were involved in metabolic processes, 7 were involved in cellular processes and signaling, 1 was involved in information storage and processing, 3 were multifunctional or general function prediction only and 8 were function unknown or no match in Genbank. One ORF was probably a novel gene since it had no match to published sequences. Other genes detected include those coding for glyoxalase I, endotoxin biosynthesis and LuxR family of transcriptional regulator. Overall, our results revealed the potential mechanism used by the bacteria as its survival, colonization and adaptation strategy coexisted in the dinoflagellate phycosphere. This study has also showed that dinoflagellate-associated bacteria community is a valuable source for discovery of novel bacteria species and novel genes and gene products.

Key Words: HAB-species, microbial community, metagenomics analysis, genes discovery.

Introduction. Over the past few decades, much of human activity such as rapid development in the agricultural sector, waste and sewage disposal activities, rapid coastal development and increasing use of fossil fuels have increased the rate of discharge of nitrogen, phosphorus and other nutrients into marine ecosystems through rivers, groundwater and the atmosphere. In the sea, these nutrients promote the reproduction of algae. When the concentration of the nutrient reaches too high, it will cause excessive growth of algae that lead to the phenomenon called harmful algal bloom (HAB red tide) (Zohdi & Abbaspour 2019). The effects of HAB can be felt by the entire coastal ecosystem including the impact on human health, food supply and recreational activities either directly or indirectly (Bechemin et al 1999; Willis et al 2018). Economic losses due to these events, while difficult to ascertain, could also be substantial.

Some HAB species can produce toxins. The toxins can cause seafood poisoning in human if intoxicated. Paralytic shellfish poisoning (PSP) is one of the notable HAB-associated seafood poisoning. The dinoflagellate from the genus of *Alexandrium* was the first dinoflagelate associated with PSP when 6 people died and causing 102 people to fall

ill in 1927 in San Francisco, USA (Kodama 2000). Since then, PSP toxins have been detected in several *in vitro* cultured dinoflagellate species and these *include Alexandrium tamarense, A. fundyense, A. tamiyavanichii, A. minutum, Pyrodinium bahamense* var *compressum* and *Gymnodinium catenatum* (Cembella 1998; Usup et al 2002). The shellfish poisoning or PSP is an ongoing problem in several regions such as America, Europe and Southeast Asia. This poisoning is due to a suite of heterocyclic guanidines, collectively referred to as saxitoxin (STX) (Danish-Daniel et al 2016). The toxins are transferred to humans through consumption of bivalve mollusks such as mussels, clams, oysters or scallops that feed on the toxic dinoflagellates and accumulate the toxin in their tissues.

Several previous studies have showed that a specific bacterial assemblage could be found in the phycosphere of dinoflagellates (Sapp et al 2007; Zhou et al 2019). The bacteria are believed to contribute to dinoflagellate physiology and toxigenesis. They may, for example, enhance metabolism, or be involved in co-metabolism processes. They may even lyse their dinoflagellate hosts. It is also possible that they produce the toxin, or a precursor to the toxin, or perhaps they are involved in degrading the toxin. These bacteria have a potentially more profound role in the development of HAB events (Uribe & Espejo 2003; Green et al 2004). In order to elucidate the actual mechanism of algae-bactria interaction, culture-dependent and culture-independent approcahes could be used. Previous studies have shown that the culture-independent metagenomic approach is particularly useful for studying microbial ecology in the natural environment (Béjà et al 2000; Rondon et al 2000; Gillespie et al 2002; Heidelberg et al 2010). Metagenomic libraries containing large-scale insert DNA such as in bacterial artificial chromosome (BAC), fosmid and cosmid libraries are particularly suitable for use to isolate large groups of genes where bioactive compounds can be obtained (Ginolhac et al 2004) or can also be used to study the physiology of uncultivated microorganisms through genetic information obtained (Moreira et al 2006).

The precise nature of the association of bacteria with dinoflagellates in culture is still not well understood. Hence, in the present study, we attempt to fully sequence and analyse a metagenomic libraries-derived fosmid clone previously constructed from bacterial community associated with toxic dinoflagellate *Alexandrium tamiyavanichii*, in order to provide insights on the potential functional role of the dinoflagellate-associated bacteria at the genomic level.

Material and Method. In this study, we selected a fosmid clone for shotgun full insert sequencing from a metagenomic library available in our laboratory. This fosmid metagenomic derived libraries was previously constructed from bacterial community associated with clonal culture of the toxic marine dinoflagellate *Alexandrium tamiyavanichii* AcMS01.

Shotgun library construction for DNA sequencing. A fosmid clone designated as MgKD02a007C11 was selected in this study. Initially, the fosmid DNA was extracted and purified using the Qiagen Large-Construct Kit (Qiagen Inc., USA) following the manufacturer's protocol. Then, DNA fragmentation was performed using Nebulizers (Invitrogen, USA) to produce DNA fragments ranging in size from 1.5 to 3 Kbp following the manufacturer's protocol. Subsequently, DNA cloning was carried out using the TOPO Shotgun Subcloning Kit (Invitrogen, USA) according to the protocol as recommended by the manufacturer. Briefly, DNA was modified by producing blunt ends using T4 DNA polymerase and Klenow polymerase (Invitrogen). Then, the DNA was ligated into the pCR4Blunt-TOPO (Invitrogen) plasmid vector for 5 minutes at room temperature. The plasmid vector with DNA insert was then electroporated into the *Escherichia coli* strain TOP10 Electrocomp competent cell (Invitrogen) using Gen-Pulser (Bio-Rad Laboratories, Inc., USA) at ~1800 V. The recombinant cells were then poured onto LB agar plates (SIGMA, St. Louis, MO) containing antibiotic kanamycin (50 µg mL^{-1}) (SIGMA, St. Louis, MO) and 40 µL X-Gal (40 mg mL^{-1}) (SIGMA, St. Louis, MO). The agar plates were then incubated at 37°C overnight. Subsequently, the white and blue colonies were transferred

to 96-wells plates containing new Luria-Bertani (LB) medium supplemented with kanamycin (50 µg mL^{-1}) and 20% (v/v) glycerol and stored at -80°C.

DNA sequencing. DNA sequencing of the recombinant plasmids was carried out with 8X coverage. Briefly, plasmids DNA were extracted and purified using Montage plasmid preparation kit (Millipore, USA) following the manufacturer's protocol. Purified plasmids were then sequenced using dideoxy chain termination (ABI Prism BigDye terminator cycle sequencing ready reaction kit, PE-ABI, USA) and universal primers T7 (5'-TAATACGACTCACTATAGGG-3') and T3 (5'-ATTAACCCTCACTAAAG-3'). Sequencing was carried out on an ABI 3730-XL automated DNA sequencer (PE Applied Biosystem Inc., USA). The generated DNA sequence was quality checked, assembled and edited using Staden Package software (Staden 1996; Staden et al 2003). Low quality DNA sequences were identified and removed using Pregap4 and the sequences were assembled using Gap4.

Bioinformatics analysis for gene prediction and annotation. The ORF (open reading frames) for the assembled contigs was predicted using Glimmer software version 3.02 (Delcher et al 2007) and ORF Finder in Artemis software package (Rutherford et al 2000; Carver et al 2008). The predicted ORFs from Glimmer and ORF Finder were then compared and edited using Artemis package software. Functional annotations for each ORF obtained were performed using several bioinformatic softwares. Among them, BLASTP (Altschul et al 1997) was used to compare the ORF obtained with the sequence from the GenBank databases. The function of each ORF was determined according to the COG category (Tatusov et al 1997, 2003). SignalP (Nielsen et al 1997; Bendtsen et al 2004) was used for signal peptide prediction and TopPred II (Claros & von Heijne 1994) was used to predict the transmembrane helices.

Results and Discussion. Several previous studies have shown that the metagenomic approach is particularly useful to elucidate microbial diversity and their functions (Béjà et al 2000; Moreira et al 2006). Fosmid-end sequencing followed by targeted shotgun sequencing was used in metagenomics study. This approach allows the discovery of novel genes and new insights of the unculturable microorganisms. In the present study, full DNA insert of the fosmid clone MgKD02a007C11 was successfully sequenced. It contains 32,976 bp nucleotide sequence with an estimated G+C content of 59.57%. A total of 26 ORFs have been successfully predicted with amino acid sequence lengths between 114 and 1107 aa. From the BLAST results, 16 ORFs showed significant sequence similarity to known gene functions, 2 ORFs showed similarities with proteins whose function was predicted in general only, 7 ORFs showed similarities with proteins whose function was unknown and 1 ORF was a gene product that did not have homologous to any protein from the GenBank database. Based on COG function, it was found that most gene products were involved in the transport and metabolism of amino acids (ORF1, ORF7, ORF16 and ORF17) and were also involved in biogenesis of cell wall/membrane/envelope (ORF8, ORF9 and ORF10). Interestingly, ORF5 was found to be no matches with any protein from the GenBank database and TopPred's analysis of this protein showed that it had a transmembrane helices domain. This suggests that ORF5 could potential to be a new protein. Most ORFs also have domains predicted as transmembrane helices indicating that their products are membrane-anchored. In addition, there were ORFs that overlapped with each other. ORF9 overlapped with ORF10 by 42 nucleotides while ORF19 overlapped with ORF20 by 10 nucleotides. About 80.8% of the DNA sequence was having similarity to bacteria *Sagitulla stellata* from the Rhodobacteraceae family. Table 1 showed each predicted ORF along with other information such as nucleotide position, amino acid size and function. Figure 1 showed the genome organization of the fosmid clone MgKD02a007C11 and the predicted protein function of each ORF according to COG category.

Table 1

Putative ORF predicted from the DNA insert sequence of fosmid clone MgKD02a007C11 and their predicted functions

ORF	*Size in aa*	*Nucleotide position*	*Predicted function*	*COG category*	*Predicted signal peptide*	*Predicted trans-membrane*
1	125	657-1031	Lactoylglutathione lyase	Amino acid metabolism and transport	No	No
2	175	1088-1612	Hypothetical protein	No functional prediction	Yes	2 trans-membranes
3	524	1634-3205	Oxidoreductase, putative	General functional prediction only	No	3 trans-membranes
4	275	3265-4089	Class II aldolase/adducin family protein	Carbohydrate metabolism and transport	No	No
5	385	4397-5551	No significant similarity found	No functional prediction	No	2 trans-membranes
6	667	5862-7862	Outer membrane autotransporter barrel	Secretion, motility and chemotaxis	Yes	9 trans-membranes
7	197	7957-8547	Twin-arginine translocation pathway signal	Amino acid metabolism and transport	Yes	1 trans-membranes
8	538	8866-10479	Peptidoglycan binding protein, putative	Cell wall structure and biogenesis and outer membrane	Yes	1 trans-membranes
9	364	10509-11600	UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase	Cell wall structure and biogenesis and outer membrane	No	3 trans-membranes
10	114	11558-11899	Acyl carrier protein, putative	Cell wall structure and biogenesis and outer membrane	No	No
11	403	11906-13114	Beta-ketoacyl synthase family protein	lipid metabolism	No	5 trans-membranes
12	269	13202-14008	Methyltransferase type II	Coenzyme metabolism	No	No
13	954	14456-17317	Glutamate-ammonia ligase adenylyltransferase	Molecular chaperones and related functions; signal transduction	No	4 trans-membranes
14	249	17612-18358	Two component transcriptional regulator, LuxR family	Signal transduction	No	2 trans-membranes
15	321	18449-19411	Extracellular solute-binding protein, family 3	General functional prediction only	No	4 trans-membranes
16	284	19413-20264	Glutamine ABC transporter permease protein	Amino acid metabolism and transport	No	5 trans-membranes
17	243	20264-20992	Amino acid ABC transporter ATP-binding protein	Amino acid metabolism and transport	No	No
18	282	21118-21963	Membrane protein, putative	No functional prediction	No	9 trans-membranes

19	166	22019-22516	Hypothetical protein	No functional prediction	No	No
20	442	22506-23831	Peptidase, M23/M37 family protein	Cell division and chromosome partitioning	No	1 trans-membrane
21	289	24034-24900	Hypothetical protein	No functional prediction	No	No
22	168	25036-25539	Alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen	Molecular chaperones and related functions	No	No
23	1107	25565-28885	Hypothetical protein	No functional prediction	No	7 trans-membranes
24	458	29055-30428	Hypothetical protein	No functional prediction	No	No
25	355	30683-31747	S-adenosylmethionine: tRNA ribosyltransferase-isomerase	Translation, including ribosome structure and biogenesis	No	2 trans-membranes
26	340	31878-32972	Hypothetical protein	No functional prediction	Yes	11 trans-membranes

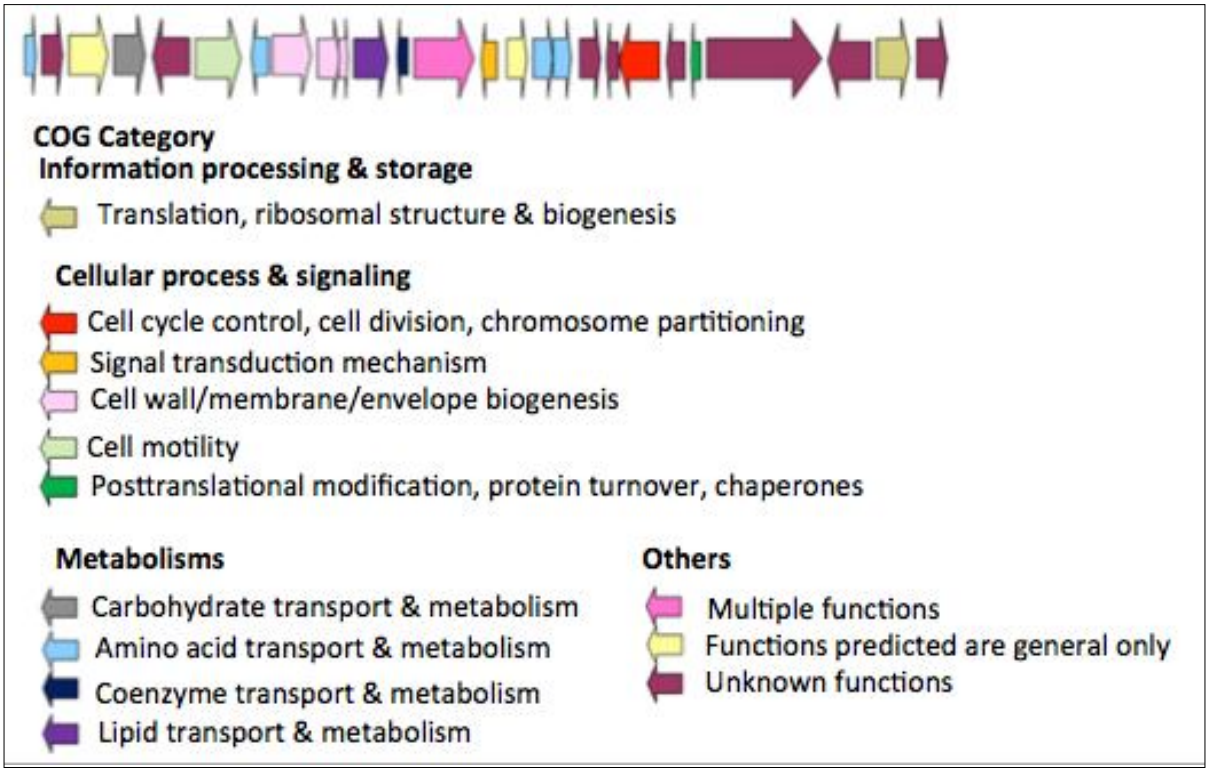

Figure 1. Genome organization for DNA insert of fosmid clone MgKD02a007C11. Each ORF was coloured according to COG category.

Analysis using conserved domain search software (Marchler-Bauer & Bryant 2004) and InterProScan (Zdobnov & Apweiler 2001) have shown that ORF1 has a domain similar with the glyoxalase I superfamily protein. Glyoxalase I (GLO1) or also known as lactoylglutathione lyase catalyzes the conversion of reactive, acyclic alpha-oxoaldehydes into the corresponding alpha-hydroxy acids (Thornalley 2003). The physiological function of GLO1 is detoxification of methylglyoxal (MG), a cytotoxic by-product of glycolysis that induces protein modification, oxidative stress, and apoptosis (Distler & Palmer 2012). In the physio-ecological aspect, methylglyoxal (MG) is important to reduce the rate of

phosphate sugar accumulation which has the effect of inhibiting growth and in turn enhances the competitiveness and survival of bacteria in the natural environment (Tötemeyer et al 1998). Thus, it is believed that the bacteria present around the dinoflagellate phycosphere use the glyoxalase I as one of the survival mechanisms. Moreover, studies showed that when under limited nitrogen conditions, methylglyoxal concentrations will increase in bacterial cells (Russell 1993; Kosmachevskaya et al 2015). It is suggested that as the nitrogen availability decreases as a result of uptake by the dinoflagellate, the associated bacteria use the glyoxalase system to prevent the accumulation of methylglyoxal in the cell as their survival and adaptation strategy.

Sequence analysis using conserved domain search software and InterProScan has shown that ORF14 encoded with a putative two-component signal transduction system or LuxR transcription regulator protein. The two-component protein is responsible for various types of biological processes in the natural environment including quorum sensing and toxin production (Li & Tian 2012). In complex communities such as in the phycosphere environment, these two-component proteins can play a significant role in determining the structure and function of a population through signaling or influencing the production of specific proteins (Girard 2019). Protein of the LuxR family is also important in gene expression (Birck et al 2002). This could be used by the bacteria to adapt to environmental changes in the phycosphere.

Another interesting predicted putative ORF from the fosmid clone in this study is the ORF20 which encodes the peptidase M23/M37 family. Many of the peptidase family displayed potent bacteriolytic activity (Grabowska et al 2015). Peptidase M23/M37 is a metalloendopeptidase used by certain bacteria to lyse the cells of other bacteria as a defense mechanism (Bochtler et al 2004). There were studies showing that peptidase from the M23/M37 family was also used by certain soil bacteria such as *Lysobacter enzymogenes* to lyse a variety of soil microorganisms including yeast, nematodes and bacteria (Chen et al 2006; Rawlings & Salvesen 2013). In this study, although the specification of the peptidase function encoded by ORF20 is still unclear, it is believed that certain bacterial species that exist around the dinoflagellate phycosphere use this peptidase as one of the mechanisms to dominate other microflora.

Conclusions. Harmful algal bloom and its associated seafood poisoning remains a global issue that should be gaining more attention. Studies on the dynamic of algal bloom formation and algal toxigenesis need to be given emphasis including the relationship between HAB species and the associated bacterial community. In the present study, we successfully sequenced and analysed the fosmid clone MgKD02a007C11 in order to provide some insights into the bacteria-algal interactions. The genome sequence of DNA insert contains ~32.9 kbp with G+C content of 59.57% consisting of 26 ORFs ranging between 114 and 1107 aa has been successfully predicted. This DNA fragment was believed to be part of the bacterial genome of the Rhodobacteraceae family. Analysis of the genome sequence revealed the potential role of the bacterial survival, colonization and adaptation strategy around the dinoflagellate phycosphere. There were 6 predicted ORFs that potentially encoded hypothetical proteins whose function was unknown and 1 ORF was a new gene whose identity and function was unknown. It is recommended that further analysis of those protein sequences via modeling and gene expression should be performed in the future to determine their exact functions. Findings of this study also suggested that bacteria associated with dinoflagellates are a valuable source for metagenomic study and such study could yield information and products useful for environmental monitoring as well as for the control of algal blooms.

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