Detection of Keto Synthase (KS) Gene Domain in Sponges and Bacterial Sponges

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Abstract

Sponges are sources of various useful natural products. The natural compounds derived from sponges are mostly complex polyketide. The polyketide synthase genes (PKS) is responsible for the biosynthesis of complex polyketides. During this research, we detected ketosynthase (KS) gene domain from 10 species of marine sponges, collected from Mediterranean Sea (*Aplysina aerophoba, Petrocia ficiformis, Axinella damicornis, Axinella veronicas, Crambe crambe)* and North Sea *(Halichondria panicea, Haliclona xena, Haliclona oculata, Suberites massa, Ephydatia fluviatilis).* We also detected KS gene domain from 12 bacterial sponge isolated from H. panacea. We used a PCR-based approach to detect the ketosynthase (KS) gene domain of Polyketosynthase (PKS) genes. Our result showed that the sponges and the bacterial sponges are mostly KS-positive (700 bp). The presence of the PKS gene indicates the ability of the sponges to produce bioactive compound and potential natural product. The presence of the KS domain in both of the sponges and the bacterial sponges might also indicate that the bacterial sponges involved in the biosintesis of secondary metabolite of the sponges.

Keywords: sponges, bacterial sponges, secondary metabolite, PKS gene, ketosynthase

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Introduction

Sponges are multicellular invertebrates from Phylum Porifera. Sponges have association with wide variety of microorganism; include bacteria, archaea, fungi and microalgae. Sponges are differentiated into two types, based on the abundance and the diversity of microbial community: High Microbial Abundance (HMA) sponges and Low Microbial Abundance (LMA). The HMA sponges biomasses consist of up to half of the microbial symbiont, meanwhile fewer symbionts were found in the biomasses of LMA sponges (Hentschel *et al.*, 2003; Hochmuth *et al.*, 2010; Giles *et al.*, 2013).

Sponges are the sources for natural products. Majority of secondary metabolites produced by marine sponge are complex polyketides, that are potentially as antimalarial, antibiotic, anti-inflammatory, antiviral, antitumor, and antifouling, including a diverse group of pharmacologically important antibiotics: erythromycin and tetracycline (Sipkema *et al.*, 2005; Taylor *et al.*, 2007; Hochmuth & Piel, 2009).

The gene that is responsible for the biosynthesis of polyketides is polyketide synthases gene (PKS). The PKS that are commonly found in the sponge metagenomes and known to be specific for the sponge is called a sup (sponge symbiont ubiquitous) PKS. The sup PKS is an unusual type of small PKS that generates methyl-branched fatty acids that are commonly present in the sponges. The other type is the trans-acyltransferase (trans AT) PKS. The trans-AT PKS belong to an evolutionarily distinct enzyme family. This type of PKS commonly found in the symbionts and also known to be present in a free-living bacteria (Fieseler *et al., 2007;* Fisch *et al.*, 2009). The complex polyketides that are normally attributed to symbionts are psymberin (Cichewicz *et al.*, 2004), onnamide A (Piel *et al.*, 2004), rhizoxin A (Piel *et al.*, 2003), and bryostatin (Sudek *et al.*, 2006). The production of these polyketides

A (Piel *et al.*, 2003), and bryostatin (Sudek *et al.*, 2006). The production of these polyketides were assigned to trans-AT PKS (Piel *et al.*, 2003).

The polyketide synthase (PKS) gene represents a prominent enzyme class that is responsible for the synthesis of complex polyketides. The polyketides are classified according to the architecture of their biosynthesis enzymes. Each of the polyketide synthases (PKS) resembles one of the classes of fatty acid synthases (FAS). During research, most of the reported PKS are type I PKS, which is large, highly modular proteins, and possess a multidomain architecture similar to the type I FAS of fungi and animals. The minimal set of most PKS modules is a ketosynthase (KS) domain, an acyltransferase (AT) domain, and an acyl carrier protein (ACP) domain. (Schirmer *et al.*, 2005; Jenke-Kodama *et al.*, 2005; Fieseler *et al.*, 2007; Ridley *et al.*, 2008).

The secondary metabolites with similar structures are biosynthesized by gene clusters that harbour certain homologous genes. The biosynthetic cluster gene for sponge polyketides can be identified using the cultivation-independent technique. A PCR based-analysis is sufficient to identify the PKS genes that are corresponds to the production of bioactive molecule (Zazopoulos *et al.*, 2003; Schirmer *et al.*, 2005; Gontang *et al.*, 2010; Hochmuth *et al., 2010*). The presences of the PKS gene domain in sponges indicate the ability of sponges to produce bioactive compound. Therefore, the purpose of our research was to study the PKS gene in sponges and sponges-associated bacteria. In this paper, we showed the detection of ketosynthase (KS) gene domain in sponges from Mediterranian Sea and North Sea, as well as in sponge-associated bacteria of *H. panicea*.

Materials and Methods

Sponges Material and Bacterial Isolates. The sponges were collected from Mediterranean Sea and North Sea. Sterile artificial seawater was used to wash the samples. Initial identification of sponges from the Mediterranean Sea by Dr. Ir. Detmer Sipkema and from the North Sea was conducted by Dr. Ir. Rene Wiffels based on their morphology. Afterwards, approximately 3 gram of the sponges samples were subsequently crushed with mortar and pestle. The cell suspension was mixed with 50% glycerol in sterile artificial seawater and was stored at -80°C until further use (Dr. Mohamad Azrul Naim, personal communication). The bacterial isolates was selected from previous research by Dr. Mohamad Azrul Naim (personal communication) based on their ability to shows the halogenase activity. The selected isolates was growth in marine broth for overnight before proceed with the genomic DNA isolation.

Genomic DNA Isolation. The genomic DNA isolation was done according to the manufacture protocols. The Fast DNA SPIN kit for soil, MP Biomedicals (for sponges sample collected from Mediterranian Sea and for the bacterial isolates) and the Qiagen Blood & Tissue Kit (for sponges sample collected from North Sea) were used to isolate the genomic DNA from sponge cell suspensions. The Nano drop (ThermoFisher Scientific, St. Leon-Rot, Germany) was used to measure the concentration of sponge genomic DNA.

PCR Amplification. The primer that were used for PCR screening was adapted from Piel (2002). The HotStart Phusion polymerase (Thermo scientific) was used in our screening. The mixture for one time PCR reaction was prepared (in total of 50 µl): 27 µl of dH_2O, 10 µl of 5×HF phusion buffer, 1 µl of dNTP mix, 2.5 µl of Forward primer (10 mM) and 2.5 µl Reverse primer (10 mM), 1.5 µl of DMSO, 0.5 µl of Taq DNA Phusion Polymerase and 5 µl of template DNA. The PCR conditions was performed as follow: Pre-denaturation 98°C (20 sec), 30 cycles of denaturation 98°C (10 sec), annealing 55°C (20 sec) and elongation 72°C (1 min) and the final extension 72°C (5 min). The second PCR was performed in 20 cycles, using 2 µl of PCR amplicon as template in total of 50 µl PCR reaction. The amplicon was visualized in 1% of agarose gel electrophoresis.

Molecular Identification of Sponge Specimens and Bacteria Based on rRNA Gene Analysis. Molecular identification of the North Sea sponges were done by amplification of partial small subunit 18S rRNA gene using universal eukaryotes primer EukF (AAC CTG

GTT GAT CCT GCC AGT) and EukR (TGA TCC TTC TGC AGG TTC ACC TAC) (Medlin *et al*., 1988). Bacteria from sponges were done by 16S rRNA gene analysis. Sponges from Mediterranean Sea were identified based on 18S rRNA gene analysis generated from the pyrosequencing reads of previous study (Dr. Mohamad Azrul Naim, personal communication). Accession numbers for the sponge 18S rRNA gene are KC899022 – KC899052 and ERS225560 – ERS 225575.

Results and Discussion

In this research we used sponges that were collected from Mediterannean Sea and North Sea. The identification of sponges was done by PCR amplification using 18S rRNA gene analysis. Each of the sponge species was prepared as cell suspensions in duplicate (*Ephydatia fluviatilis* and *Crambe crambe)* or triplicate (*Aplysina aerophoba, Petrocia ficiformis, Axinella damicornis, Axinella veronicas, Halichondria panicea, Haliclona xena, Haliclona oculata, Suberites massa, Ephydatia fluviatilis).* Table 1 showed the sponges that we used during this experiment.

Research on the LMA sponges (*Amphimedon compressa*, *Niphates digitalis, Ptilocaulis* sp. and *Callyspongia vaginalis*) suggested that the LMA sponge is generally not the major source of complex polyketides (Hochtmuth *et al*., 2010). In this research, we are able to detect the presence of KS gene domain among sponges using the KS primer derived from Piel (2002). A PCR-strategy was used during our experiment to detect the presence of ketosynthase (KS) gene domain among samples. Our result (Figure 1) showed that most of the sponges are KS-positive (amplicon size approximately 700bp), except the sponge *Suberitas massa* 2 (lane 11) and *S. massa* 3 (lane 12). However, the sponge *S. massa* 1 (lane 10) shown a KS-positive, therefore we thought that the negative result might be because of not enough DNA sample to be amplified.

Table 1. Sponge samples

No.	Sponge Name	Abbr.	Location Collected
1	*Aplysina aerophoba* 1	AA1	Mediterranean Sea
2	*Aplysina aerophoba* 2	AA2	Mediterranean Sea
3	*Aplysina aerophoba* 3	AA3	Mediterranean Sea
4	*Petrocia ficiformis* 1	PF1	Mediterranean Sea
5	*Petrocia ficiformis* 2	PF2	Mediterranean Sea
6	*Petrocia ficiformis* 3	PF3	Mediterranean Sea
7	*Axinella damicornis* 1	AD1	Mediterranean Sea
8	*Axinella damicornis* 2	AD2	Mediterranean Sea
9	*Axinella damicornis* 3	AD3	Mediterranean Sea
10	*Axinella verrunicosa* 1	AV1	Mediterranean Sea
11	*Axinella verrunicosa* 2	AV2	Mediterranean Sea
12	*Axinella verrunicosa* 3	AV3	Mediterranean Sea
13	*Crambe crambe* 5	Cr5	Mediterranean Sea
14	*Crambe crambe* 6	Cr6	Mediterranean Sea
15	*Halichondria panicea* I	HpeI	North Sea
16	*Halichondria panicea* II	HpeII	North Sea
17	*Halichondria panicea* III	HpeIII	North Sea
18	*Haliclona xena* I	HxeI	North Sea
19	*Haliclona xena* II	HxeII	North Sea
20	*Haliclona xena* III	HxeIII	North Sea
21	*Haliclona oculata* I	HoeI	North Sea
22	*Haliclona oculata* II	HoeII	North Sea
23	*Haliclona oculata* III	HoeIII	North Sea
24	*Suberites massa* 1	Sm1	North Sea
25	*Suberites massa* 2	Sm2	North Sea
26	*Suberites massa* 3	Sm3	North Sea
27	*Ephydatia fluviatilis* 1 (10x)	Ef1	North Sea
28	*Ephydatia fluviatilis* 2 (10x)	Ef2	North Sea

Microbial communities in marine sponges contribute to more than 40% of the sponge biomass. Studies on sponge-associated bacteria covering different phyla, such as: Gammaproteobacteria (Jayatilake *et al*., 1996), Firmicutes (Devi *et al*., 2010), and Proteobacteria (Tabares *et al*., 2011). Along within this research, Dr. Mohamad Azrul Naim (personal communication) isolated sponge-associated bacteria from marine sponge *H. panicea*. From those bacterial sponges, we choose 12 isolates (Table 2) that shows halogenase activity. The identification of bacterial isolates was done by PCR amplification using 16S rRNA gene analysis. From this bacterial isolates we aim to detect the presence of the KS gene domain in bacterial sponges by using PCR strategy. The result showed in Figure 2.

A similar result as Figure 1 is shown in Figure 2, where most of the songe-associated bacteria isolated from *H. panicea* were KS-positive (amplicon size approximately 700bp), except for isolate No. 113 (lane 3, *Bacillus* sp.), Isolate No. 118 (lane 7, *Halomonas* sp.), Isolates No. 124 (lane 11, Alpha Proteobacteria*)* and Isolates No. 125 (lane 12, *Nocardioides alkalitolerans).*

The production of secondary metabolites by sponge is a common phenomenon since most of the antitumor compounds were isolated from marine sponges. However, diverse natural products of sponges are localized in the associated bacterial cells. Therefore, the bacterial symbionts are proposed as the producers of invertebrate drug candidates (Piel, 2002; Gao & Huang, 2009; Schneemann *et al*., 2010). Some of the sponge-associated microbes are specific to the host and involved in the production of secondary metabolites (Piel *et al*., 2004a; Kennedy *et al*., 2007; Taylor *et al.,* 2007). Study on marine sponge *Theonella swinhoei* provides evidence that the symbiotic bacteria are the key contributors to the polyketide chemistry of diverse eukaryotic hosts (Piel *et al.,* 2004b; Fieseler *et al*., 2007; Hochmuth *et al.,* 2010). Kotterman *et al*. (2003) investigated *H. panicea* and shown that the sponge produce a neuroactive compound and brominated compound. Research by Schneemann and co-workers (2010) showed a high number of different secondary metabolites isolated from actinobacteria, which associated with *H. panicea*. Extensive investigation on actinomycetes associated with *H. panicea* showed a high antimicrobial activity. Further research by Bondu and co-workers (2012) had isolated antiviral and cytotoxic compounds from *C. crambe*. The compounds crambescin (previously called crambin) and crambescidin (Jares-erijman *et al.,* 1991) is the chemotaxonomic marker for *C. crambe*. Henceforth, *H. panicea* and *C. crambe* is interesting sponges from natural product perspective to be screened for the presence of the polyketide synthase (PKS) genes.

The molecular screening of PKS genes can be used as a primary selection for the production of bioactive compound among the sponge candidates because the PCR-based screening of KS gene in a genome with high number of biosynthetic gene clusters mostly will give a positive result. The PKS is a giant protein, which consist of repeated modules. Each module carries a set of catalytic domains for chain extension and modification. The KS domain is present in each of the PKS modules. As the most conserved region, the KS domain is adequate to investigate the diversity of PKS genes. The KS applicant might only represent a small fraction of the PKS gene clusters within the sponge metagenomes, but it is sufficient for further phylogenetic analysis to identify a diverse set of PKS genes (Schirmer *et al*., 2005; Fieseler *et al*., 2007; Schneemann *et al*., 2010).

Our research shown that the KS gene domain were present in most of sponge samples and sponge-associated bacteria, indicated by the amplicon size of KS gene domain which around 700 bp. However, our result only shows that the sponges and sponge-associated bacteria are probably potential to produce bioactive secondary metabolite. Further experiment using more advance technology such as pyrosequencing is necessary to get a high-througput result. Pyrosequencing result can be used to study the phylogenetic and the diversity of PKS gene in sponges and sponge-associated bacteria. A clear understanding about the PKS gene in sponge and sponge-associated bacteria will reveal the symbiothic mechanism of sponge and sponge-associated bacteria, especially in the biosynthesis of secondary metabolite.

Table 2. Sponge-associated bacteria of *Halichondria panicea*

Lane	Isolate No	Identified as
1	111	*Paracoccus sp.*
2	112	*Enterovibrio sp.*
3	113	*Bacillus sp.*
4	114	*Paracoccus sp.*
5	115	*Micrococcus sp.*
6	117	*Gamma proteobacterium*
7	118	*Halomonas sp.*
8	119	*Bacillus pocheonensis*
9	120	*Bacillus licheniformis*
10	122	*Pseudovibrio sp.*
11	124	*Alpha proteobacterium*
12	125	*Nocardioides alkalitolerans*

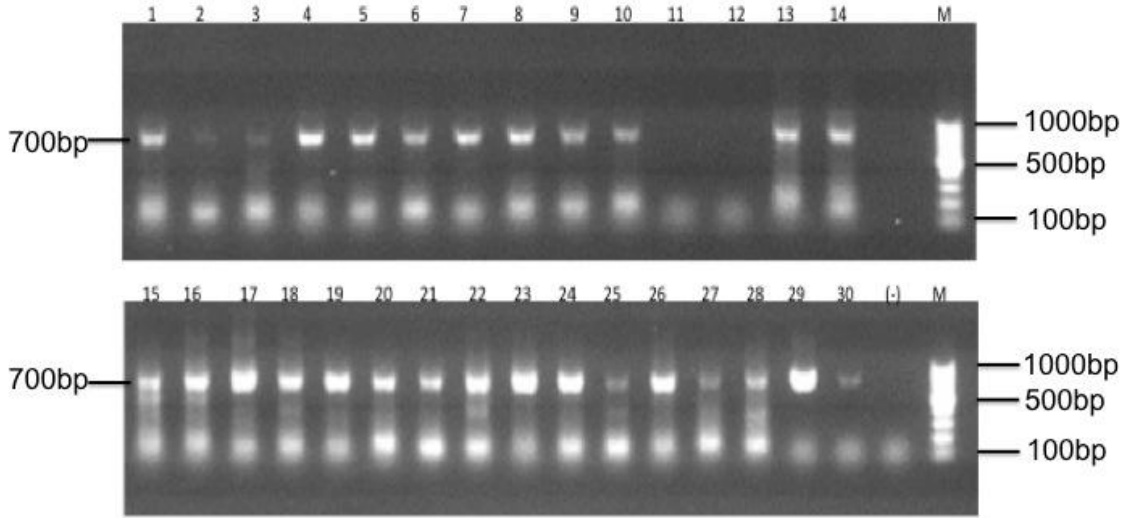

Figure 1. The result of PCR screening. Lane 1-3: *Halichondria panicea*, lane 4-6: *Haliclona xena*, lane 7-9: *Haliclona oculata*, lane 10-12: *Suberitas massa*, lane 13-15: *Aplysina aeropoba*, lane 16-18: *Petrocia ficiformis*, lane 19-21: *Axinella verrunicosa*, lane 22-24: *Axinella damicornis*, lane 25-26: *Crambe crambe*, lane 27-28: *Ephydatia fluviatilis*, lane 29-30: clone 122, marker (M): 100bp

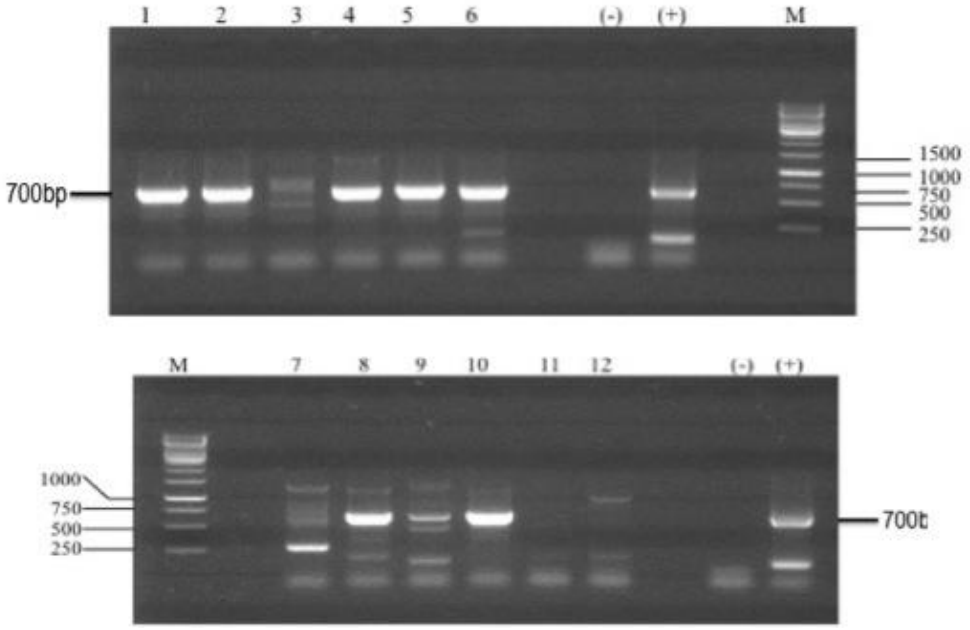

Figure 2. The PCR result of sponge-associated bacteria of *H. panicea*, marker (M): 1kbp plus

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