



Quantification of polyketide synthase genes in tropical urban soils using real-time PCR



Thai-Hoang Le^a, Vaishnavi Sivachidambaram^a, Xinzhu Yi^a, Xu Li^b, Zhi Zhou^{a,*}

^a Department of Civil and Environmental Engineering, National University of Singapore, 117576, Singapore

^b Department of Civil Engineering, University of Nebraska-Lincoln, Lincoln, NE 68588, United States

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ABSTRACT

Polyketide synthases (PKSs) catalyze the biosynthesis of polyketides and may contribute to the natural production of antibiotics and pose selective pressure for the development of antibiotic resistant bacteria in the environment. Although conventional PCR have been developed to detect the presence of PKS genes, no previous studies have been done to quantify the abundance of PKS genes in environmental samples. In this study, two sets of degenerate real-time PCR (qPCR) primers (PKS1-F/PKS1-R, PKS2-F/PKS2-R) with high specificity and sensitivity were developed to quantify PKS type I and type II genes. These primers were subsequently used to quantify PKS genes in tropical urban soils, and both PKS genes were widely detected in all soil samples. The absolute abundance of PKS type I ranged from 1.7×10^6 to 4.7×10^6 copies per gram of soil and the absolute abundance of PKS type II genes ranged from 2.4×10^5 to 1.5×10^6 per gram of soil, and the abundance of PKS type I gene was consistently higher than that of PKS type II gene. The relative abundance of PKS type I gene was positively correlated with that of PKS type II gene ($p < 0.01$). Regression analyses indicate that PKS gene abundance was negatively correlated with environmental factors, such as selected antibiotics, sulfate, and metals ($p < 0.05$), but was not correlated with land use type. The studies on the correlation between environmental factors and PKS genes could provide useful information to understand natural production of antibiotics and its associated environmental risks.

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1. Introduction

Antibiotics in the environment may exert a selective pressure for the development of antibiotic resistance, which has been considered as a global public health concern and has received wide research interests (Esiobu et al., 2002; Levy, 1998; Schnabel and Jones, 1999). The development of antibiotic resistant bacteria, especially in environmental microbial communities, is believed to result from the exposure to bioactive antibiotics even at very low concentrations and persistence of antibiotics in the environment (Huerta et al., 2013). Although human medicines and veterinary drugs have been considered as the main sources for antibiotics in the environment, biosynthesis of natural antibiotics by indigenous microorganisms may also contribute to antibiotics in natural environment, especially in natural soil samples (Hu et al., 2010; Huerta et al., 2013; Ji et al., 2012; Sarmah et al., 2006). Evaluation of biosynthesis of natural antibiotics may help us understand the sources and health risks of antibiotics in the environment.

Polyketides are carbon backbones of a large family of structurally diverse natural products possessing a wide range of antibiotic and other pharmaceutical properties (Katz and Donadio, 1993; Kealey et al., 1998; Tsoi and Khosla, 1995). The diversity of polyketides derives from various ways of constructing and folding of the carbon chains, which are assembled into natural products through a series of reactions. Polyketides are catalyzed by polyketide synthases (PKSs). PKS type I is a modular multifunctional protein catalyzing the biosynthesis of macrolide antibiotics, such as erythromycin and tylosin, and it contains many different active sites and each site is required for an enzyme-catalyzed step in the carbon-chain assembly and modification pathway (Hutchinson, 1995; Paitan et al., 1999; Staunton and Weissman, 2001; Tsoi and Khosla, 1995; Zucko et al., 2007). PKS type II is a monofunctional protein catalyzing the biosynthesis of aromatic polyketides, such as 6-methylsalicylic acid and actinorhodin, and it contains a single set of iterative active sites that perform the reaction repeatedly to build the entire carbon-chain backbone (Staunton and Weissman, 2001; Tsoi and Khosla, 1995). Since the biosynthesis of many antibiotics is catalyzed by PKSs that are encoded by PKS genes, the quantification of PKS type I and PKS type II genes can be used to evaluate the likelihood of natural antibiotic production in environmental samples.

However, only few studies have been done to detect and quantify the abundance of PKS genes. Traditionally, membrane-based method

* Corresponding author. Tel.: +1 765 496 3559.

E-mail address: zhizhou@purdue.edu (Z. Zhou).

¹ Present address: School of Civil Engineering and Division of Environmental and Ecological Engineering, Purdue University, 550 Stadium Mall Drive, West Lafayette, IN 47907, United States.

of DNA–DNA hybridization was used to detect specific genes, but this method has a low sensitivity and the abundance of target genes has to be above 10^5 to be detected (Beller et al., 2001). PCR was developed to detect low levels of target genes (Saiki et al., 1988). Conventional PCR has been used to detect the presence/absence of PKS genes in environmental and food samples (Ayuso-Sacido and Genilloud, 2004; Geisen et al., 2004; Metsa-Ketela et al., 1999; Moffitt and Neilan, 2001) and a fungus species (Amnuaykanjanasin et al., 2005), but it is not suitable for the quantification of PKS genes because conventional PCR amplicon sizes are too large (>500 bp). Furthermore, conventional PCR-based methods can be easily affected by many factors, such as reagent depletion during the amplification of target DNA for end-point detection and PCR biases caused by competition between PCR amplicons and primers during template annealing (Becker et al., 2000; Suzuki et al., 2000). Real-time PCR (qPCR) can overcome this problem by quantifying PCR amplicons at the exponential phase of DNA reactions, and have advantages of high sensitivity, high specificity, less time consumption, and more accuracy in a complex DNA population (Becker et al., 2000; Hristova et al., 2001; Takai and Horikoshi, 2000). Recently, several sets of qPCR primers were developed to quantify PKS genes involved in biosynthesis of chaetoglobosin in a fungus *Chaetomium globosum* (Hu et al., 2012) and ochratoxin in a fungus *Aspergillus carbonarius* (Atoui et al., 2007). However, these qPCR primers were used to target PKS genes in individual fungal strains and therefore they are not suitable to detect bacterial PKS genes in environmental samples because many species exist in natural environment and bacteria and fungi have separate groups of PKS genes (Kroken et al., 2003).

The first objective of this study was to develop two sets of degenerate qPCR primers to detect and quantify PKS type I and II genes in multiple microorganisms. The second objective was to measure the occurrence and abundance of PKS type I and type II genes in tropical urban soils. We hypothesized that our newly designed qPCR primers could be used to quantify PKS genes in environmental soil samples, and PKS genes were widely distributed in tropical urban soils. The correlation between PKS gene abundance and land use types, antibiotic concentrations, and other environmental factors was also evaluated. The information in this study allows us to better understand biosynthesis and potential health risks of natural antibiotics in the environment.

2. Materials and methods

2.1. Sampling locations

Soil samples were collected from ten locations covering five land use types (residential, industrial, open space, park, and agricultural) in Singapore, a tropical country located at Southeast Asia with an average temperature between 24.8 °C and 31.2 °C throughout the year. The sampling sites were selected adjacent to rivers and canals, but these rivers and canals were not affected by discharged wastewater as Singapore directly discharges its treated wastewater to the sea. Site A is an agricultural land near a flower farm. Site I1 is an industrial land near a canal. Site I2 is the other industrial land near a wetland. Site O is an open space beside a natural reservoir. Site P is a park near a lake. Sites R1, R2, R3, R4, and R5 are residential areas beside rivers or canals.

2.2. DNA preparation

DNA was extracted from both pure cultures and soil samples for PCR experiments. The positive control strain, *Streptomyces albus* (*S. albus*, DSM 40313), contains both PKS type I and PKS type II genes (DeHoff et al., 1999) and was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Braunschweig, Germany). *S. albus* was cultivated with DSMZ Medium 65 (glucose 4 g/L, yeast extract 4 g/L, malt extract 10 g/L, CaCO₃ 2 g/L) at 28 °C and shaken at 150 rpm for 3 days before DNA extraction. The negative control strains, *Blautida producta* (*B. producta*, DSM 2950) and

Methanococcus maripaludis (*M. maripaludis*, DSM 14266), were also purchased from DSMZ (Braunschweig, Germany). *B. producta* was cultivated with DSMZ Medium 78 and *M. maripaludis* was cultivated with DSMZ Medium 141. Total genomic DNA of *S. albus*, *B. producta*, and *M. maripaludis* was extracted using an UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, CA, USA). Total genomic DNA of soil samples was extracted using a Power Soil DNA Isolation Kit (MoBio Laboratories, CA, USA) following the manufacturer's protocol. 1 g of soil was extracted for 100 µl of total DNA and triplicate extractions were performed. The concentrations of DNA from pure cultures and soil samples were measured in NanoDrop ND-1000 (BioFrontier Technology Co., Germany), and stored at –20 °C. To ensure DNA quality during extraction process, conventional PCR amplification was performed with universal 16S rRNA gene primer, as previously described (Fierer et al., 2005). The presence of the 16S rRNA gene was confirmed in all DNA samples through DNA electrophoresis and UV visualization.

2.3. Primer design

Two sets of degenerate primers (PKS1-F/PKS1-R, PKS2-F/PKS2-R) were designed in this study to detect and quantify PKS type I and PKS type II genes, respectively. Because PKS genes are variable and multiple oligonucleotides can encode the same amino acid sequences in syntheses, primer design was based on amino acid sequences. All PKS type I and PKS type II amino acid sequences were downloaded as FASTA files from the National Center for Bioinformatics (NCBI, <http://www.ncbi.nlm.nih.gov/>) on 30th May 2013. A total of 320 PKS type I and 15 PKS type II amino acid sequences (Table S1 and Table S2 in the Supporting Information) were aligned using the MUSCLE tool in the software MEGA (Tamura et al., 2011) to identify the most conserved regions in these sequences. Then aligned files were uploaded into the online program iCODEHOP to make blocks of conserved amino acids as previously described (Rose et al., 2003). A series of degenerate primers were selected and the best primer sets with low degeneracy and high specificity were selected with relatively small amplicon sizes for a high efficiency in qPCR analysis. Finally, the designed primers were sent for synthesis by AIT Biotech (Singapore). The details of primers PKS1-F/PKS1-R and PKS2-F/PKS2-R are listed in Table 1.

2.4. Optimization of PCR conditions and comparison with previous primers

PCR conditions were optimized for the newly designed primers. Conventional PCR experiments were conducted in a Veriti 96-Well Fast Thermal Cycler (Applied Biosystem, CA, USA). Each conventional PCR reaction contained 2 µl 10× Faststart PCR buffer (Roche, IN, USA), 0.25 mM dNTPs (Promega, Singapore), 0.0375 unit/µl Taq Faststart DNA polymerase (Roche, IN, USA), 0.5 mM forward primers (AITBiotech, Singapore), 0.5 mM reverse primers (AITBiotech, Singapore), and 250 ng genomic DNA template, and the final volume was adjusted to 20 µl with diethylpyrocarbonate (DEPC)-treated distilled deionized water (PureLab Option, Elga, Singapore). PCR amplifications were carried out with the following settings: 95 °C for 5 min; repeated 40 times with a cycle of denaturation at 95 °C for 30 s, annealing at annealing temperature for 30 s, elongation at 72 °C for 30 s, and finally 72 °C for 10 min. The annealing temperature for PKS type I and PKS type II primers was optimized by running conventional PCR experiments with a temperature gradient from 58 to 66 °C. A positive control strain (*S. albus*) that contains PKS genes and two negative control strains (*B. producta* and *M. maripaludis*) that contain no PKS genes were used in the conventional PCR experiments. A negative control experiment with distilled deionized water instead of DNA template was also conducted to confirm the absence of primer dimers, hairpin products, and other potential contaminations.

After optimization, primers PKS1-F/PKS1-R and PKS2-F/PKS2-R were compared with two previously reported conventional PCR primers targeting PKS type I and PKS type II genes. One set of primers, K1F/M6R,

Table 1
Characteristics of PCR primers used in this study.

Primers	Primer sequence (5'–3')	Suitable for quantification	Degeneracy	Annealing temperature (°C)	Target gene/microorganisms	Amplicon size (bp)	References
PKS1-F/PKS1-R	CGG GGC ACC GCC ATS AAC MAS GRC G/CGC CCA GCG GGG TGS CSG TNC CGT G	Yes	16/16	62	PKS type I/bacteria	201	This study
PKS2-F/PKS2-R	CCA CCC GCT ACG SSB HCC ACA/CGT TCT GCT TGG TGC CGS WNC CGT GSG C	Yes	36/32	64	PKS type II/bacteria	147	This study
K1F/M6R	TSA AGT CSA ACA TCG GBC A/CGC AGG TTS CSG TAC CAG TA	No	12/4	55	PKS type I/ <i>Actinomyces</i>	1200–1400	Ayuso-Sacido and Genilloud (2004)
540F/1100R	GGI TGC ACS TCI GGI MTS GAC/CCG ATS GCI CCS AGI GAG TG	No	96/32	64	PKS type II/ <i>Actinomyces</i>	554	Wawrik et al. (2005)
Eub338/Eub518	ACT CCT ACG GGA GGC AGC AG/ATT ACC CGG GCT GCT GG	Yes	0/0	55	16S rDNA/bacteria and archaea	200	Fierer et al. (2005)

was designed by Ayuso-Sacido et al. to detect PKS type I genes with the following PCR conditions: 5 min at 95 °C, 35 cycles for 30 s at 95 °C, 2 min at 55 °C, 4 min at 72 °C, and finally 10 min at 72 °C (Ayuso-Sacido and Genilloud, 2004). The other set of primers, 540F/1100R, was designed by B. Wawrik et al. to detect PKS type II genes with the following PCR conditions: 5 min at 95 °C, 40 cycles for 1 min at 95 °C, 1 min at 64 °C, 1.5 min at 72 °C, and finally 15 min at 72 °C (Wawrik et al., 2005).

2.5. PCR product analysis

PCR products were analyzed with DNA electrophoresis. 9 µl PCR products were mixed with 1.5 µl 6× DNA loading dye (Thermo Scientific, USA) and loaded into a gel with 2% molecular agarose (Invitrogen, USA), 0.5% TAE buffer, and 0.01% GelRed Nucleic Acid (Biotium, USA). The gel was run at 100 V for 45 min and visualized under a UV transilluminator in E-gel Imager (Life Technologies, CA, USA). Purity of amplified PCR products was examined through a melting curve analysis following qPCR reactions. At temperature lower than melting temperature of PCR products, the double-stranded DNA status allowed SYBR Green to bind on and turn on its fluorescence. When temperature reached to T_m, the double-stranded DNA denatured and released SYBR Green and turned off its fluorescence. Purity of the PCR products was checked with the melting curve plots. PCR products amplified using PKS1-F/PKS1-R and PKS2-F/PKS2-R were isolated from an agarose gel band and then purified through a PCR purification kit (Qiagen, USA) and analyzed through a sequencing analysis with capillary electrophoresis method performed by AITBiotech (Singapore). The DNA sequencing results were confirmed by blasting on NCBI GenBank.

2.6. qPCR calibration curves and quantification of PKS genes

To build calibration curves, PKS type I and PKS type II genes were amplified using primers PKS1-F/PKS1-R and PKS2-F/PKS2-R and 16S rRNA gene was amplified using primers EUB338/518 with extracted DNA of *S. albus* in a conventional PCR. Ten-fold dilution series were prepared to build standard curves for qPCR. Triplicate samples were used for each dilution. qPCR experiment was performed in a StepOnePlus Real-Time PCR system (Applied Biosystem, CA, USA) following the standard protocol provided by Applied Biosystem. Each qPCR reaction contained 10 µl SYBR Select Master Mix (Applied Biosystem, CA, USA), 0.25 µl 10 mM forward primers, 0.25 µl 10 mM reverse primers, 2 µl DNA template, and 7.6 µl DEPC-treated distilled deionized water. qPCR reactions were carried out with the following settings: holding at 50 °C for 2 min and 95 °C for 2 min; amplification for 40 times with a cycle of 95 °C for 15 s and annealing and elongating at 60 °C for 1 min, and finally melting curve analysis at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The amplification profiles for each series of DNA dilutions during 40 cycles were recorded. The threshold line was automatically defined within the logarithmic increase phase of the fluorescence data by the StepOnePlus software (Applied Biosystem, CA,

USA). A threshold cycle (C_t) value was determined as the cycle number where fluorescence data crossed the threshold line. Calibration curves were built by plotting the threshold C_t values versus the logarithm of initial gene copy numbers. The gene copies of PKS type I and PKS type II genes were calculated as previously described (Ausubel et al., 1992). The molecular weight of a gene was determined by multiplying the size of a gene in base pairs and the average molecular weight of a double-stranded DNA base pair (649 Da). Absolute gene abundance was calculated as gene copies normalized to the weight of each soil sample, and relative gene abundance was calculated as percentage of PKS genes normalized to the abundance of 16S rRNA gene.

2.7. Measurement of antibiotics

Top layer soils (<10 cm) were collected with grass and plant roots removed using a methanol-rinsed shovel, and then filled in methanol-rinsed wide mouth polyethylene bottles. At each sampling site, soil samples were collected from three different locations and homogeneously mixed. Soil samples were stored on ice and transferred to laboratory in dark. All soil samples were processed and stored based on the United States Environmental Protection Agency (EPA) guideline (Englert, 2007). Blanks were run along each sampling process to make sure positive detection was not from contamination.

A total of six antibiotics were analyzed in this study, which include four macrolide antibiotics (azithromycin, clarithromycin, erythromycin, tylosin), one lincosamide antibiotic (lincomycin), and one chloramphenicol antibiotic (chloramphenicol). All antibiotics and Supel HLB solid phase extraction (SPE) cartridges (reversed phase, 20 µm PE frit, 60 mg, 3 ml) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isotope-labeled surrogates were used as internal standards. Erythromycin (N, N-dimethyl-¹³C₂) and (+/–)-chloramphenicol (ring-d₄, benzyl-d₁) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Individual stocks and calibration solutions were prepared in methanol and stored at –20 °C in dark. Ammonium acetate and HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). EDTA was purchased from Tokyo Chemical Industry (Tokyo, Japan). Laboratory reagent water was prepared in a water purification system from ELGA Labwater (Wycombe, UK).

Sample preparation procedure followed EPA method 1694 but sample extraction conditions (pH 2 or pH 7) were optimized for each antibiotic. The recommended sample extraction condition in EPA Method 1694 was acidic, but our results indicated that macrolides were recovered more efficiently under neutral extraction condition. Therefore, neutral extraction was used in EPA Method 1694 for this study. Spiking recovery experiments were conducted to evaluate the accuracy and precision of the method and matrix effect was evaluated under different extraction conditions. HPLC was performed on an Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA) and separations were performed on a reversed phase with a 50 mm × 2.1 mm column packed with 2.7 µm particles (Agilent Poroshell 120 SB-C18). Mass

analysis was carried out using Agilent Model 6490 mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with electrospray Agilent Jet Stream technology.

2.8. Measurement of nutrients

Soil samples were air dried and crushed in a mortar and sieved through a 1.0 mm pore diameter sieve. Inorganic ions were extracted from soils via aqueous extraction as previously described (Jackson, 2006). 2 g dried soil samples were mixed with 20 ml deionized water, sonicated for 30 min using an ultrasonicator (Cole-Parmer, Chicago, IL, USA), and centrifuged and filtered through a 0.20 μm PTFE syringe filter. The soil extracts were injected to ion chromatography for ion analysis. Procedure blanks was prepared in every batch to detect contamination. Ions were analyzed using an IC system (ICS 3000, Dionex, USA). Fluoride (F^-), chlorine (Cl^-), nitrite (NO_2^-), nitrate (NO_3^-), sulfate (SO_4^{2-}) and phosphorous (PO_4^{3-}) were analyzed with a column IonPac AS11-HS (Dionex) coupled with a guard column AG12A (Dionex). Ammonia (NH_4^+) was analyzed with a column IonPac CS12A (Dionex) coupled with a guard column AG12A (Dionex). Sample preparation and analysis were done in duplicates.

2.9. Measurement of metals

Metals were extracted from soils using microwave assisted acid digestion following EPA method 3051 (3051A, U.-E.M., 2007). 0.5 g dried and grinded soil samples were weighted in perfluoroalkoxy alkanes (PFA) digestion vessels and mixed with 10 ml concentrated nitric acid. The digestion vessels were sealed and placed in the carousel in the ETHOS One microwave digestion system (Shelton, CT, USA) according to the manufacturer's recommendation. The temperature of each sample was increased to 175 $^\circ\text{C}$ in less than 5.5 min and kept at 175 $^\circ\text{C}$ for 10 min. After cooled to room temperature, the digestates were diluted 20 times in a 50 ml centrifuge tube with deionized water. The diluted digestates were centrifuged and filtered through 0.20 μm membrane syringe filters. Sample preparation and analysis were done in duplicates. The filtrates were analyzed using the Optima 7300 DV Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) system (PerkinElmer, Waltham, MA, USA). The analysis process followed the US EPA method 6010C (6010C, U.E.M., 2007). A total of 14 metal elements were tested: sodium (Na), magnesium (Mg), potassium (K), calcium (Ca), chromium (Cr), manganese (Mn), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), cadmium (Cd), gallium (Ga), lead (Pb), and arsenic (As). A multi-element standard was purchased from Merck (Darmstadt, Germany). Calibration blanks were prepared by acidifying reagent water to the same concentrations of the acids in the samples (3.5% nitric acid). Method blanks were run along each sampling process. Spiking experiments at concentrations of 0.02 and 0.2 mg/g dry wt. were conducted to evaluate the accuracy of the analysis.

2.10. Statistical analysis

All reported data are shown as average values of measurements. Standard deviations for triplicate measurements or half-range for duplicate measurements were reported as error bars. Pearson correlation and Spearman correlation analyses were conducted between PKS gene abundance, six environmental antibiotics, seven nutrients, and 14 metals. Correlation was considered significant with a p value of <0.05 .

3. Results

3.1. Specificity and efficiency of primers PKS1-F/PKS1-R and PKS2-F/PKS2-R

The optimal annealing temperature for PKS1-F/PKS1-R was identified as 62 $^\circ\text{C}$ and the optimal annealing temperature for PKS2-F/PKS2-R was identified as 64 $^\circ\text{C}$. Under the optimized PCR conditions, PKS

type I and PKS type II genes were successfully detected by PKS1-F/PKS1-R and PKS2-F/PKS2-R (Fig. 1) and the PCR product was specific to the positive control strain *S. albus*. Only one single band of PCR product was observed in the agarose gel for PKS type I and PKS type II amplicons with the extracted DNA of *S. albus*. Moreover, no bands were detected in the agarose gel for negative controls without DNA template or with the extracted DNA of positive control strains *B. producta* and *M. maripaludis*. These results suggest that the newly designed PKS primers in this study can be used to specifically amplify PKS genes. Additionally, strong bands for all three tested strains were detected with the EUB338/518 primers targeting the 16S rRNA gene, which confirmed the quality of extracted DNA as well as the efficiency of the PCR experiments.

The comparison results of the primers indicate that PKS1-F/PKS1-R and PKS2-F/PKS2-R were similar or more efficient than previously reported primers K1F/M6R and 540F/1100R to target PKS genes in environmental samples (Fig. 2). The expected amplicon sizes of primer sets K1F/M6R, 540F/1100R, PKS1-F/PKS1-R, and PKS2-F/PKS2-R were 1200–1400 bp, 560 bp, 201 bp, and 147 bp, respectively (Table 1). For PKS type I gene, single and strong bands at the expected amplicon size were observed for *S. albus* and two environmental soil samples using primers PKS1-F/PKS1-R, while multiple bands were observed with previously reported primers K1F/M6R. For the PKS type II gene, both PKS2-F/PKS2-R and previously reported primers 540F/1100R showed single bands at expected sizes for all three tested samples, and a stronger band was obtained with PKS2-F/PKS2-R for *S. albus*. For the two soil samples, slightly weaker bands were detected with the PKS2-F/PKS2-R primers, which may be related with the amplicon size of PKS2-F/PKS2-R primers, which is about four times smaller than that of 540F/1100R, and less amount of nucleotides in the double-strand DNA may result in less UV absorbance and low intensities. Additional bands for primers 540F/1100R were detected at the bottom of the electrophoresis graph, which were possibly primer dimers. The amplicon size of PCR products of primers 540F/1100R was 560 bp. Although a large amplicon size may give a bright band under UV light, it is usually not suitable for quantification with qPCR analysis. Primers PKS1-F/PKS1-R and PKS2-F/PKS2-R were designed with smaller amplicon sizes (201 bp and 147 bp) that can be used efficiently in qPCR to quantify the absolute gene copy number of PKS genes.

The specificity of the primers PKS1-F/PKS1-R and PKS2-F/PKS2-R was also examined by performing sequencing analysis for their PCR products. Since *S. albus* was known to possess the PKS genes, its extracted DNA was used as DNA template for the PCR amplification. After a single band at the expected size on the agarose gel was confirmed for each primer, the PCR products were purified and sequenced. The sequences of the PKS type I and PKS type II amplicons were blasted on the NCBI

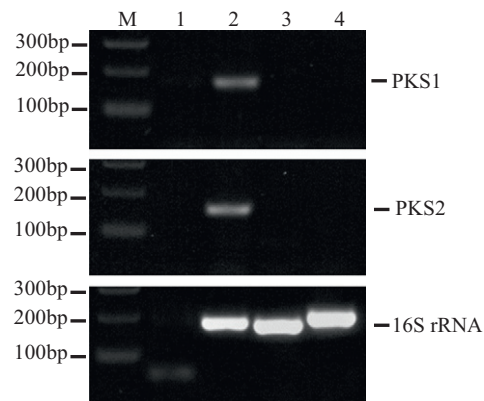


Fig. 1. Specificity of PKS1-F/PKS1-R and PKS2-F/PKS2-R primers in conventional PCR. Lane M: 100 bp DNA marker; lane 1: negative control with DNA template; lane 2: positive control with *Streptomyces albus*; lane 3: negative control with *Blautida producta*; lane 4: negative control with *Methanococcus maripaludis*.

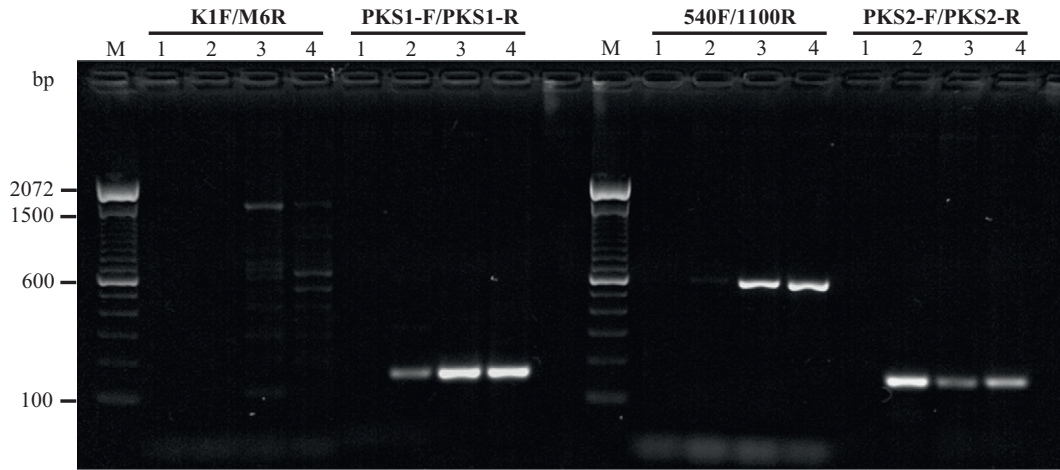


Fig. 2. Comparison between PKS1-F/PKS1-R and PKS2-F/PKS2-R primers and previously reported primers K1F/M6R and 540F/1100R. Lane M: 100 bp DNA marker; lane 1: negative control without DNA template; lane 2: *S. albus*; lane 3: soil sample #1; lane 4: soil sample #2.

GenBank, and almost all matched hits with high scores were identified as PKS type I and PKS type II genes (Table S3). The PKS type I gene with the highest matched score was identified as *S. albus*, while that of PKS type II gene was identified as uncultured bacterium clone due to limited sequences of PKS type II gene in the NCBI database. These results validated the feasibility of using primers PKS1-F/PKS1-R and PKS2-F/PKS2-R to amplify PKS genes.

3.2. Accuracy of qPCR

The calibration curves in qPCR were constructed for PKS type I gene, PKS type II gene, and 16S rRNA gene (Fig. S1). The calibration curves indicate that the relationship between C_t values and the gene copy numbers was negatively correlated with regression equations as $C_t = 43.906 - 4.158 \log_{10}(\text{gene copy})$ for PKS type I gene ($R^2 = 0.999$), $C_t = 43.398 - 4.435 \log_{10}(\text{gene copy})$ for PKS type II gene ($R^2 = 0.994$), and $C_t = 47.525 - 4.388 \log_{10}(\text{gene copy})$ for 16S rRNA gene ($R^2 = 0.998$). The PCR products were confirmed on an agarose gel. The linear correlation and high regression coefficients indicated that primers PKS1-F/PKS1-R and PKS2-F/PKS2-R were accurate to quantify PKS genes in qPCR experiments. In the melting curve analysis, there was only one peak for each gene with the melting temperature at 90.5 °C for PKS type I gene, 91 °C for PKS type II gene, and 87.5 °C for 16S rRNA gene (Fig. S2), and the results indicated that these primers bound specifically to DNA templates so that only one PCR product was generated. The detection limit of the PKS type I and PKS type II genes evaluated in this calibration curve ranged from 10^4 to 10^9 gene copies/ μl .

3.3. Occurrence of PKS genes in tropical urban soils

As shown in Fig. 3, both PKS type I gene and PKS type II gene were widely detected in all soil samples. The absolute abundance of PKS type I ranged from 1.7×10^6 to 4.7×10^6 copies/g soil and the absolute abundance of PKS type II genes ranged from 2.4×10^5 to 1.5×10^6 copies/g soil. On average, the abundance of PKS type I gene was 5.8-fold higher than that of PKS type II gene. Another interesting observation is the co-occurrence of PKS type I and PKS type II genes and the relatively high gene abundance of PKS type I gene, which has not been reported in previous studies. A linear correlation ($p < 0.01$) was detected on the relative abundance of PKS type I gene and PKS type II in soil samples (Fig. 4), although such a correlation was not detected in the absolute gene abundance of PKS type I and PKS type II genes.

3.4. Correlation between PKS genes with land use type, antibiotic concentrations, and environmental factors

There was no clear correlation between gene abundance and land use type of soil samples. Among the ten sampling locations, the three

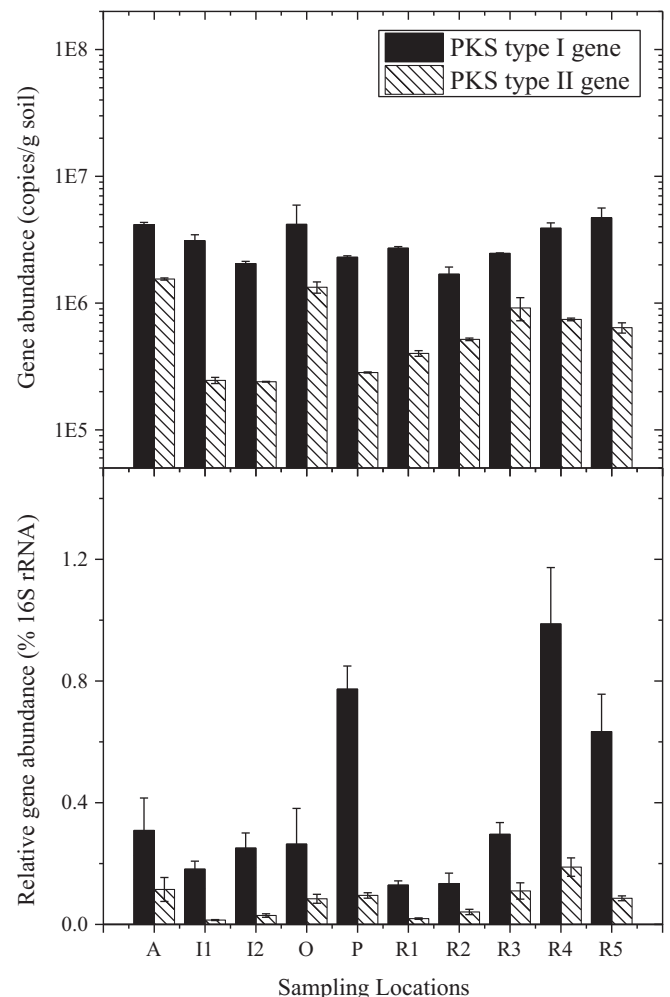


Fig. 3. Absolute and relative abundance of PKS genes in soil samples.

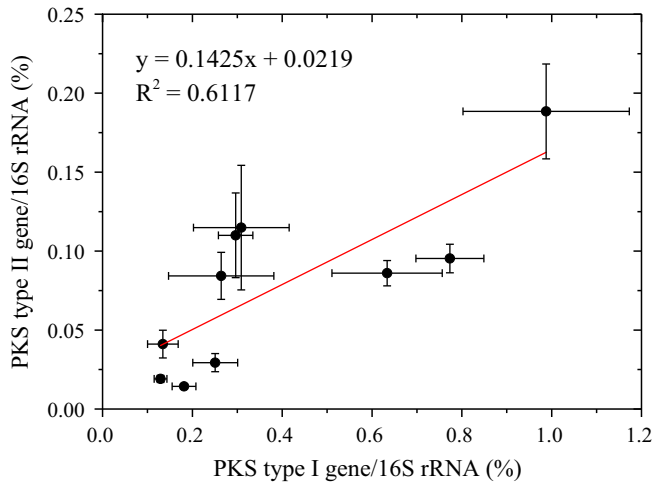


Fig. 4. Correlation between relative abundance of PKS type I gene and PKS type II gene in soil samples.

highest absolute gene abundance levels of PKS type I gene were found in locations R5 (residential land), O (open space), and R4 (residential land), and the three highest relative gene abundance levels were found in locations R4 (residential land), P (park), and R5 (residential land). Conversely, the three highest absolute gene abundance levels of PKS type II gene were found in locations A (agricultural land), O (open space), and R3 (residential land), and three highest relative gene abundance levels were found in locations R4 (residential land), R3 (residential land), and A (agricultural land). The levels of PKS genes in residential lands were generally high. Although we expected that PKS gene levels were high among soils in industrial lands, our results

indicate that the levels of PKS genes in industrial lands (I1, I2) were among the lowest in our study.

No correlation was detected between PKS gene abundance and natural antibiotics, except that tylosin was negatively correlated with relative abundance of PKS type I gene and chloramphenicol was negatively correlated with absolute abundance of PKS type II gene (Table 2). Tylosin is a macrolide antibiotic and the biosynthesis of tylosin could be catalyzed by PKS type I, and chloramphenicol is an aromatic antibiotic and the biosynthesis of chloramphenicol could be catalyzed by PKS type II (Abraham and Newton, 1960; Glazko et al., 1950; Kanfer et al., 1998).

The correlations between PKS genes and six environmental antibiotics, seven nutrients, and 14 metals were also investigated (Table 2). Among the 27 tested environmental factors, 9 and 18 factors were significantly correlated with PKS genes in Pearson's correlation and Spearman's correlation analyses, respectively. The results indicate that no correlation was detected between PKS gene abundance and nutrients, except that sulfate was positively correlated with relative abundance of PKS type I gene and negatively correlated with absolute abundance of PKS type II gene. These unique correlations may enable PKS genes as potential markers for environmental sulfate salt, a nutrient factor that is well linked to other environmental factors (Duffy and Défago, 1999; Ohge et al., 2003). Additionally, the absolute abundance of PKS type I gene was negatively correlated with Mg and K, and relative abundance of PKS type I gene was negatively correlated with Ni and Cu. Conversely, PKS type II gene was negatively correlated with Na, Mg, K, Ca, Cr, Mn, Co, Ni, and Cu.

4. Discussion

This study has revealed, for the first time, the wide occurrence of PKS genes in tropical urban soils. The 100% detection frequency of PKS genes

Table 2
Correlation between absolute and relative PKS gene abundance and environmental factors in soil samples. Values are correlation coefficients (p-values) in Pearson and Spearman correlation analyses. Significant ($p < 0.05$) correlation coefficients and their corresponding p-values are marked with underlines.

Environmental factors	PKS1 gene copy		PKS1 gene/16S (%)		PKS2 gene copy		PKS2 gene/16S (%)	
	Pearson	Spearman	Pearson	Spearman	Pearson	Spearman	Pearson	Spearman
Antibiotics								
Erythromycin	0.407 (0.243)	0.176 (0.627)	0.219 (0.543)	-0.079 (0.829)	-0.264 (0.461)	-0.455 (0.187)	-0.183 (0.613)	-0.467 (0.174)
Tylosin	-0.305 (0.392)	-0.398 (0.255)	-0.417 (0.231)	<u>-0.701 (0.024)</u>	-0.260 (0.469)	-0.182 (0.616)	-0.461 (0.180)	-0.45 (0.192)
Clarithromycin	-0.140 (0.699)	-0.058 (0.873)	-0.316 (0.373)	-0.522 (0.122)	-0.219 (0.542)	-0.174 (0.631)	-0.385 (0.272)	-0.406 (0.244)
Azithromycin	-0.140 (0.699)	-0.058 (0.873)	-0.316 (0.373)	-0.522 (0.122)	-0.219 (0.542)	-0.174 (0.631)	-0.385 (0.272)	-0.406 (0.244)
Lincomycin	0.479 (0.161)	0.304 (0.393)	0.142 (0.697)	0.045 (0.901)	-0.212 (0.557)	-0.433 (0.211)	-0.167 (0.645)	-0.433 (0.211)
Chloramphenicol	-0.149 (0.681)	-0.225 (0.532)	-0.312 (0.380)	-0.355 (0.315)	-0.460 (0.181)	<u>-0.683 (0.029)</u>	-0.525 (0.119)	-0.623 (0.054)
Nutrients								
F ⁻	-0.287 (0.422)	-0.176 (0.627)	-0.392 (0.262)	-0.273 (0.446)	-0.015 (0.967)	-0.091 (0.803)	-0.266 (0.458)	-0.285 (0.425)
Cl ⁻	-0.367 (0.297)	-0.176 (0.627)	-0.342 (0.333)	-0.164 (0.651)	-0.275 (0.442)	-0.345 (0.328)	-0.483 (0.157)	-0.285 (0.425)
NO ₂ ⁻	0.339 (0.338)	0.297 (0.405)	-0.087 (0.812)	0.055 (0.881)	0.406 (0.245)	0.406 (0.244)	0.079 (0.828)	0.188 (0.603)
NO ₃ ⁻	0.222 (0.538)	0.115 (0.751)	0.088 (0.809)	0.127 (0.726)	0.152 (0.675)	0.152 (0.676)	0.104 (0.774)	0.236 (0.511)
SO ₄ ⁻	-0.273 (0.478)	-0.183 (0.637)	<u>0.752 (0.020)</u>	0.333 (0.381)	-0.401 (0.285)	<u>-0.717 (0.030)</u>	0.203 (0.600)	-0.15 (0.700)
NH ₄ ⁺	-0.080 (0.825)	-0.055 (0.881)	-0.281 (0.431)	-0.236 (0.511)	-0.228 (0.526)	-0.333 (0.347)	-0.359 (0.308)	-0.248 (0.489)
PO ₄ ³⁻	0.349 (0.323)	0.236 (0.511)	-0.104 (0.774)	-0.006 (0.987)	0.617 (0.057)	0.345 (0.328)	0.143 (0.693)	0.079 (0.829)
Metals								
Na	-0.413 (0.236)	-0.503 (0.138)	-0.222 (0.537)	-0.188 (0.603)	<u>-0.652 (0.041)</u>	<u>-0.879 (0.001)</u>	-0.555 (0.096)	-0.491 (0.150)
Mg	-0.507 (0.135)	<u>-0.648 (0.043)</u>	-0.242 (0.501)	-0.382 (0.276)	<u>-0.635 (0.048)</u>	<u>-0.927 (0.000)</u>	-0.529 (0.116)	<u>-0.636 (0.048)</u>
K	-0.617 (0.058)	<u>-0.721 (0.019)</u>	-0.106 (0.771)	-0.37 (0.293)	<u>-0.694 (0.026)</u>	<u>-0.915 (0.000)</u>	-0.498 (0.143)	-0.588 (0.074)
Ca	-0.264 (0.461)	-0.067 (0.855)	-0.257 (0.473)	-0.103 (0.777)	-0.585 (0.076)	<u>-0.806 (0.005)</u>	-0.564 (0.089)	-0.600 (0.067)
Cr	-0.247 (0.491)	-0.176 (0.627)	0.021 (0.953)	-0.042 (0.907)	<u>-0.645 (0.044)</u>	<u>-0.648 (0.043)</u>	-0.460 (0.181)	-0.467 (0.174)
Mn	-0.313 (0.379)	-0.200 (0.580)	-0.268 (0.454)	-0.297 (0.405)	-0.548 (0.101)	<u>-0.745 (0.013)</u>	-0.524 (0.120)	-0.552 (0.098)
Co	-0.271 (0.448)	-0.067 (0.855)	-0.358 (0.310)	-0.515 (0.128)	-0.626 (0.053)	-0.624 (0.054)	<u>-0.669 (0.034)</u>	<u>-0.782 (0.008)</u>
Ni	-0.472 (0.168)	-0.588 (0.074)	-0.483 (0.158)	<u>-0.661 (0.038)</u>	<u>-0.709 (0.022)</u>	<u>-0.806 (0.005)</u>	<u>-0.750 (0.012)</u>	<u>-0.782 (0.008)</u>
Cu	-0.357 (0.311)	-0.394 (0.26)	-0.528 (0.116)	<u>-0.709 (0.022)</u>	-0.575 (0.082)	-0.624 (0.054)	<u>-0.712 (0.021)</u>	<u>-0.745 (0.013)</u>
Zn	-0.119 (0.743)	-0.176 (0.627)	-0.170 (0.640)	-0.37 (0.293)	-0.491 (0.149)	-0.406 (0.244)	-0.353 (0.317)	-0.406 (0.244)
Cd	-0.250 (0.487)	-0.11 (0.762)	-0.572 (0.084)	-0.485 (0.155)	-0.177 (0.625)	-0.291 (0.415)	-0.538 (0.109)	-0.562 (0.091)
Ga	-0.302 (0.396)	-0.224 (0.533)	-0.180 (0.618)	-0.212 (0.556)	-0.234 (0.516)	-0.127 (0.726)	-0.225 (0.532)	-0.164 (0.651)
Pb	0.034 (0.926)	0.03 (0.934)	-0.087 (0.810)	-0.43 (0.214)	-0.548 (0.101)	-0.527 (0.117)	-0.396 (0.257)	-0.624 (0.054)
As	-0.556 (0.095)	-0.494 (0.147)	-0.312 (0.379)	-0.363 (0.303)	-0.492 (0.149)	-0.619 (0.056)	-0.508 (0.134)	-0.488 (0.153)

in soils suggests that indigenous bacteria may commonly harbor biosynthesis genes to synthesize antibiotics to gain growth advantages over substrates and nutrients in natural environment, which are consistent with previous findings on the natural production of natural antibiotics that describe antibiotics as a chemically heterogeneous group of organic, low-molecular-weight compounds produced by microorganisms to inhibit the growth or metabolic activities of other microorganisms (Davies, 1990; Raaijmakers and Mazzola, 2012; Thomashow et al., 1997).

Although co-occurrence of PKS type I and PKS type II genes was observed, the gene abundance of PKS type I gene was consistently higher than that of PKS type II gene, which may be related with its diversity in polyketide synthesis. Although both PKS type I and PKS type II belong to the same group of enzymes catalyzing the synthesis of polyketides, they are basically different in structure and function. While PKS type II are multienzyme complexes that contain a single set of iteratively acting activities and involve in a biosynthesis of some aromatic polyketides (e.g., tetracenomycin), PKS type I are multifunctional enzymes including many modules and each module possesses a set of non-iteratively acting activities for the catalysis of one cycle of polyketide chain elongation as exemplified in biosynthesis of reduced polyketides, such as macrolides (e.g., erythromycin), polyethers and polyenes (Shen, 2003; Staunton and Weissman, 2001).

The lack of correlation between PKS genes with land use type and antibiotic concentrations may be explained by fate and transport of antibiotics in soil samples. As Singapore directly discharges its treated wastewater to the sea and urban soils are not affected with treated wastewater, antibiotics detected in soil samples are likely the results of naturally produced antibiotics from indigenous bacteria. One possible explanation for the lack of positive correlation between tylosin and PKS type I gene and between chloramphenicol and PKS type II is that PKSs are multi-enzyme complexes involved in the biosynthesis of many antibiotics with similar structures, such as macrolide antibiotics and aromatic antibiotics, and therefore the abundance of PKS genes in tested soil samples may involve in the natural biosynthesis of some other macrolides and aromatic antibiotics that compete the production of tylosin and chloramphenicol. In addition, some physical and chemical factors in natural soil environment may have some impact on the expression of PKS genes and degradation of the antibiotics. Tylosin was reported with a half-life of 4.4 to 8 days in soil environment (Carlson and Mabury, 2006; Schlüsener and Bester, 2006) and chloramphenicol with a half-life of 1 to 4.5 days (Berendsen et al., 2013; Wongtavatchai et al., 2004). The short half-lives of these two compounds suggest their low persistency in environmental soils, which might also explain the lack of positive correlation between their environmental occurrence and PKS gene abundance. Besides the stability, antibiotics have various degrees of sorption affinity on soils that could also influence their environmental fate. For example, the log K_{ow} value of chloramphenicol is 1.14 (Wongtavatchai et al., 2004), indicating little sorption affinity to soil particles. The low log K_{ow} value suggests that chloramphenicol would be more likely to be flushed to streams than to be adsorbed to soils during surface runoff events. Therefore, the chemical properties can be used to explain lack of correlation between the occurrence of PKS genes and levels of antibiotics in soil samples in this study.

The correlation between PKS genes and environmental factors may be related with statistical methods and the complexity of soil samples. It is interesting that more significant correlations were identified with Spearman's correlation analyses for the correlation between PKS genes and environmental factors. Pearson's correlation was used to measure linear relationship between normally distributed variables, while Spearman's correlation was used to measure monotonic relationship without the assumption of normal distribution of tested data. Because natural environments are open systems and gene abundance could be affected by many factors, the assumptions of linear relationship and normal distribution may not be accurate, and therefore Spearman's

correlation may reveal more statistical relationships than Pearson's correlation. In addition, many studies found that the co-occurrence of antibiotics and metals resulted in the associated resistance of microorganisms to both antibiotics and metals in the environment (Baker-Austin et al., 2006; Dhakephalkar and Chopade, 1994; Ji et al., 2012; Seiler and Berendonk, 2012). The mechanism of the associated resistance is possibly a result of two separate resistant pathways of antibiotics and metals, or a unique resistant mechanism on the antibiotic-metal complex whose activity and characteristics may vary upon the combination between antibiotics and metals (Chen and Huang, 2009, 2011), while the complex may also change its physical and chemical properties (e.g., solubility) and lead to failure in the sample measurement procedure (e.g., sample processing step), but the quantification of PKS gene was not affected. The mechanisms on the negative correlations between PKS genes and metals deserve further investigation.

In this study, two new degenerate primers were designed for qPCR to detect and quantify PKS type I and type II genes in a broad range of environmental bacteria in soils. The sensitivity, specificity, and efficiency of detecting the PKS type I and type II genes in the soil samples of our newly designed primers were compared with two existing primers in the literature using conventional PCR. The new primer was better than the existing primer to target PKS type I gene, and similar to the existing primer to target PKS type II gene. The suitability of using these primers on other bacteria could be further tested in future studies. In spite of the limited number of the tested samples, both PKS type I gene and PKS type II gene were commonly detected in all soil samples, suggesting that natural production of antibiotics is a common phenomenon in urban soils. No correlation was observed between PKS gene abundance and land use type of soil samples, but negative correlation was detected between PKS gene abundance and some environmental factors. Further studies on how environmental factors may affect the development of PKS genes would be helpful to understand the natural production of antibiotics with PKS genes and be helpful for a comprehensive environmental risk assessment of natural antibiotics in the environment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.mimet.2014.08.010>.

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