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Isolation of a Pigment-producing Strain of *Staphylococcus kloosii* from the Respiratory Tree of *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt 1835) from Malaysian Waters

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Abstrak: *Staphylococcus kloosii*, sejenis bakteria yang menghasilkan pigmen berwarna oren, telah dipencilkan dari pohon respirasi *Holothuria (Mertensiothuria) leucospilota* (Brandt 1835) dari Teluk Nipah, Pulau Pangkor, Perak, Malaysia. Laporan ini merupakan dokumentasi pertama tentang strain Gram-positif ini yang dikenali sebagai Strain 68 di Malaysia. Satu jujukan separa gen 16S ribosom RNA strain mesofilik tersebut telah didaftarkan dengan GenBank (National Center for Biotechnology Information, US National Library of Medicine) dengan nombor akses JX102547. Analisis-analisis filogenetik menggunakan kaedah hubungkait jiran serta kaedah persamaan maksimum seterusnya menyokong pengecaman strain tersebut iaitu Strain 68 sebagai *S. kloosii*. Strain berbentuk bulat ini menghasilkan pigmen-pigmen keorenan di atas agar ekstrak tripton glukosa yis (TGYEA) dan di dalam bubur nutrisi (NB) pada pH lebih kurang 7. Spektrum-spektrum nampak ekstrak-ekstrak ethanol dan methanol pigmen strain bakteria tersebut dianggap serupa dengan λ_{max} pada 426, 447 dan 475 nm dan λ_{max} pada 426, 445 dan 473 nm, masing-masing. Kedua-dua spektrum nampak kelihatan menyamai spektrum-spektrum nampak lutein, karotenoid yang bernilai komersil; walau bagaimanapun, analisis-analisis lanjutan adalah diperlukan untuk mengesahkan identitinya. Dari segi komposisi pigmen, ekstrak methanol pigmen intrasel tersebut terdiri daripada sekurang-kurangnya tiga bahan pigmen iaitu bahan pigmen oren (bahan utama), bahan pigmen kuning (paling tidak berpolar) dan bahan pigmen merah jambu (paling berpolar). Menariknya, penemuan-penemuan ini juga merupakan dokumentasi pertama tentang komposisi pigmen strain *S. kloosii* memandangkan tiada rekod berkenaan dapat dijumpai sehingga kini.

Kata kunci: *Staphylococcus kloosii*, *Holothuria (Mertensiothuria) leucospilota* (Brandt 1835), Gen 16S Ribosom RNA, Analisis-analisis Filogenetik, Pigmen Keorenan

Abstract: *Staphylococcus kloosii*, an orange pigment-producing bacterium, was isolated from the respiratory tree of *Holothuria (Mertensiothuria) leucospilota* (Brandt 1835) from Teluk Nipah, Pangkor Island, Perak, Malaysia. This report is the first documentation of this Gram-positive strain, referred to as Strain 68 in Malaysia. A partial 16S ribosomal RNA gene sequence of the mesophilic strain has been registered with GenBank (National Center for Biotechnology Information, US National Library of Medicine) with accession number JX102547. Phylogenetic analysis using the neighbour-joining method further supported the identification of Strain 68 as *S. kloosii*. The circular strain produced orange pigments on tryptone glucose yeast extract agar (TGYEA) and in nutrient broth (NB) at approximately pH 7. The visible spectra of ethanolic and methanolic pigment extracts of the bacterial strain were considered identical with λ_{max} at 426, 447 and 475 nm and λ_{max} at 426, 445 and 473 nm, respectively. Both visible spectra resemble the visible spectra of lutein, which is a commercial carotenoid; however, further analyses are required to confirm

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the identity of this pigment. The methanolic extracts of the intracellular pigments comprised at least three pigment compounds: an orange pigment compound (major compound), a yellow pigment compound (the least polar) and a pink pigment compound (the most polar). These findings are the first documentation of the pigment composition of *S. kloosii* as no such record could be found to date.

Keywords: *Staphylococcus kloosii*, *Holothuria (Mertensiothuria) leucospilota* (Brandt 1835), 16S Ribosomal RNA Gene, Phylogenetic Analysis, Orange Pigments

INTRODUCTION

Phylum Echinoderm is a large group of marine animals with a worldwide distribution. Sea cucumber (Echinodermata: Holothuroidea) is among the most popular echinoderms in Malaysia (Kamarudin *et al*. 2010b). Nearly 80 species of sea cucumber can be found in Malaysian marine environments (Kamarudin *et al.* 2010a, 2010b, 2009). *Timun laut*, *bat*, *balat*, *trepang*, *brunok*, *gamat*, and *hoi sum* (or *hai shen*) are among the local names for sea cucumber in Malaysia. Sea cucumbers are economically important in Malaysia for two reasons: first, they are used in traditional medicine (e.g., *gamat* lipid and water extracts) as well as modern medicine in Peninsular Malaysia (West Malaysia), and second, they are an important source of food in Sabah (East Malaysia).

Approximately 142 studies pertaining to Malaysian sea cucumbers were recorded until the end of the year 2011 (Kamarudin 2011). However, few studies were performed to investigate the existence and association of microorganisms or microbes, including pigment-producing strains, with Malaysian sea cucumbers, thus leading to the current study. One of these studies was performed by Farouk *et al*. (2007), in which 30 bacterial strains were isolated from *Holothuria (Halodeima) atra* Jaeger, 1833 from Malaysian waters, and 7 strains showed moderate antibacterial activity against *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*.

In this study, *Holothuria (Mertensiothuria) leucospilota* (Brandt, 1835) was chosen considering its higher level of abundance in the marine environment of Malaysia. The local species may contain indigenous microbes that help it continue to adapt and exist in various conditions. It can be found on the sandy sea floor or below the rocks in the seawaters. As a well-known *timun laut*, it is thought to be the most abundant species in Malaysia (Kamarudin *et al*. 2011). The English name of this soft-bodied species is 'white threads fish', and it is locally known as *bat puntil* or *lintah laut*. It has a long, black, tubular body, often with a reddish background. Its mouth is surrounded by tentacles and it has a posterior terminal anus (Kamarudin *et al*. 2011). Ridzwan *et al*. (2003) suggested the potential of a water extract from *H. leucospilota* as an alternative analgesic drug. *H. leucospilota* has also been proposed as a natural antioxidant with anticancer properties (Osama *et al*. 2009).

In the present study, a pigment-producing strain of *Staphylococcus kloosii*, Strain 68, was isolated from the respiratory tree of *H. leucospilota*. This finding is considered as the first documentation of *S. kloosii* in Malaysia. *S. kloosii* was previously isolated from the skin of various wild animals and only rarely from

that of farm animals (Schleifer *et al*. 1984). *S. kloosii* (Firmicutes => Bacilli => Bacillales => Staphylococcaceae => Staphylococcus => *Staphylococcus kloosii*) is a Gram-positive bacterium that grows under aerobic conditions, and its pathogenicity is unknown to date. Its colonies can be pigmented or non-pigmented. However, its pigment has not been described in detail in terms of its composition. Hence, this study further aimed to determine the genetic profile and role of this pigment-producing strain of *S. kloosii* isolated from the respiratory tree of *H. leucospilota* and to determine its microbial pigment composition by thin layer chromatography (TLC).

MATERIALS AND METHODS

Study Site

Specimens of *H. leucospilota* were collected from Teluk Nipah, Pangkor Island, Perak, Malaysia. Three individuals were sampled. The samplings took place over approximately two days, from 8–9 November 2011 (Tuesday and Wednesday). No fixed or standard sampling hours were allocated. The documentation and collection were performed during low tide. A global positioning system (GPS) was used to mark and record the position of the sampling site (not shown specifically). For short-term storage, fresh specimens of sea cucumbers were stored in ice boxes containing seawater or ice cubes during sampling. In the laboratory, specimens were transferred into a freezer for long-term storage with proper cataloguing.

Culture Media and Cultivation

A small piece of tissue from the respiratory tree of each *H. leucospilota* specimen was cut with a sterile blade and placed on tryptone glucose yeast extract agar [TGYEA (Fluka Analytical, Sigma-Aldrich, St. Louis, Missouri; ingredients: casein enzymatic hydrolysate, 5 g/l; yeast extract, 3 g/l; glucose, 1 g/l; agar, 15 g/l)] at pH 7.19 (Fig. 1). The agar plates were incubated at 37°C. After overnight incubation, the bacterial colonies were observed, and colonies with different morphologies were isolated and streaked onto new TGYEA plates. Every single colony was repeatedly subcultured onto fresh TGYEA to purify the target bacterium. The single colonies were observed under a dissecting microscope to examine their morphological characteristics. The characteristics observed were optical density, shape, colour, edge, elevation and texture of the single colonies. After Gram staining, each microscope slide containing a stained single colony was observed under a Nikon ECLIPSE (Melville, New York) 80i digital compound microscope (Fig. 1) with 1000x total magnification [the total magnification resulted from the eyepiece (10x) and the objective lens (100x)].

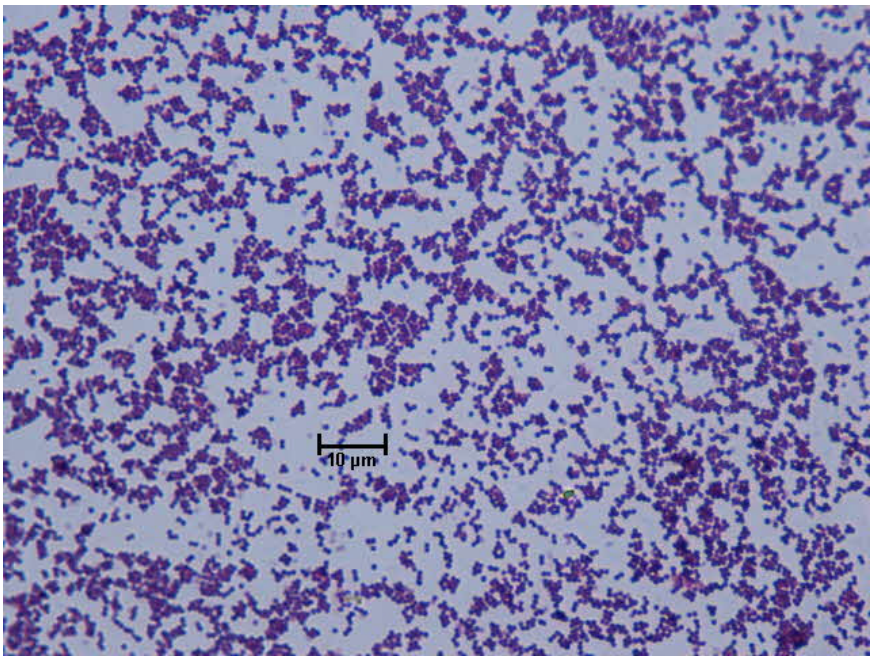

Figure 1: Gram-stained bacterial Strain 68 observed under the Nikon ECLIPSE 80i digital compound microscope with 1000x magnification. The strain was cultured for 16 hours prior to the Gram staining. The violet or purple colour resulting from Gram staining indicates that Strain 68 is a Gram-positive bacterium. Its bacterial shape is spherical or coccus.

Total Genomic DNA Extraction

Total genomic DNA (tgDNA) was extracted from all isolated bacteria using the modified cetyl trimethyl ammonium bromide (CTAB) method described by Grewe *et al*. (1993) coupled with the Geneaid Genomic DNA Mini Kit (New Taipei City, Taiwan) [blood/cultured cells]. Approximate yields of tgDNA as well as its quantity and quality were determined by electrophoresis on a 1% agarose gel using ethidium bromide as a gel stain.

Polymerase Chain Reaction (PCR)

For bacterial identification, two universal primers termed PB36 (forward primer) and PB38 (reverse primer) were used for the isolation of the 16S ribosomal RNA (rRNA) gene using an Eppendorf Mastercycler (Hamburg, Germany) gradient thermocycler. The expected length of the amplified PCR product was approximately 1.5 kb.

PB36 (forward): 5'–AGRGTTTGATCMTGGCTCAG–3' (20 bases)
PB38 (reverse): 5'–GKTACCTTGTTACGACTT–3' (18 bases)

Standard PCRs were performed using a 50 µl reaction volume containing 33.75 µl of sterilised dH_2O, 5.0 µl of 10X PCR reaction buffer, 3.0 µl of magnesium chloride (25 mM), 2.5 µl of each universal primer (5 µM), 1.0 µl of

dNTP mix (10 mM), 2.0 µl of the DNA extract and 0.25 µl of 5 U/µl *Taq* DNA polymerase. A master mix was used for amplifying a large number of samples. The cycle parameters were 5 min at 95°C for the initial denaturation followed by 29 cycles of 45 s at 95°C for denaturation, 90 s at an optimised temperature (e.g., 55°C) for annealing, and 1 min 30 s at 72°C (60 s/kb) for extension prior to a 7 min extension step at 72°C with a final hold at 4°C. Approximate yields of amplified DNA as well as its quantity and quality were determined by electrophoresis on a 1% agarose gel with ethidium bromide as a gel stain.

PCR Product Purification and DNA Sequencing

A Geneaid Gel/PCR DNA Fragment Extraction Kit (New Taipei City, Taiwan) was used for direct purification of the PCR products. Purified PCR products in suspension form were prepared prior to sending samples for sequencing. Sequencing was performed using the BigDye® Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) [ACGT]. The cycle sequencing reaction was performed in a programmable cycler (Tpersonal Combi Thermocycler, Biometra GmbH, Goettingen, Germany). The cycle sequencing reaction was performed for 35 cycles of 96°C for 10 s, 55°C for 5 s, and 60°C for 4 min prior to a holding step. The reactions were then precipitated with ethanol and sodium acetate. The rapid thermal ramp was 1°C/s. Sequencing was performed using an ABI 377 automated sequencer (PE Applied Biosystems, Foster City, California).

Phylogenetic Analyses

In this study, the Basic Local Alignment Search Tool program (BLAST; retrieved at http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine the presence of *S. kloosii* among the isolated bacterial strains based on their 16S rRNA gene sequences. *S. kloosii* Strain 68 was selected for further analyses as it is a pigment-producing bacterial strain that has not been previously reported in Malaysia, and no record of the *S. kloosii* pigment composition could be found to date. Chromas Lite (version 2.01, Technelysium Pty Ltd., Queensland, Australia) was used to display the results of fluorescence-based DNA sequence analyses. Multiple sequence alignment of the forward reaction sequences was performed using ClustalX (version 2.1) [Thompson *et al*. 1997], and the sequences were subsequently aligned by eye. Molecular Evolutionary Genetics Analysis 5 (MEGA5) [Tamura *et al*. 2011] was subsequently used to reconstruct a phylogenetic tree using the neighbour-joining method (Saitou & Nei 1987) [Fig. 2]. The phylogenetic analysis was performed with 97 nucleotide sequences (Table 1). All positions containing gaps and missing data were eliminated. There were a total of 1319 positions in the final dataset. Phylogenetic confidence was estimated by bootstrapping (Felsenstein 1985) with 1000 replicate data sets. The optimal tree with a branch length sum of 0.30220933 is shown. The tree was drawn to scale, and the branch length units are the same as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method (Tamura & Nei 1993), and the units are the number of base substitutions per site. Estimates of evolutionary divergence (Table 2) and percent nucleotide composition (Table 3)

of the partial 16S ribosomal RNA gene sequences of Strain 68 and other members of *S. kloosii, Staphylococcus spp.* and the Strain 68 cluster were also calculated. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. TreeView (Win32) version 1.6.6 (Page 1996) was used to display and edit the reconstructed phylogenetic trees.

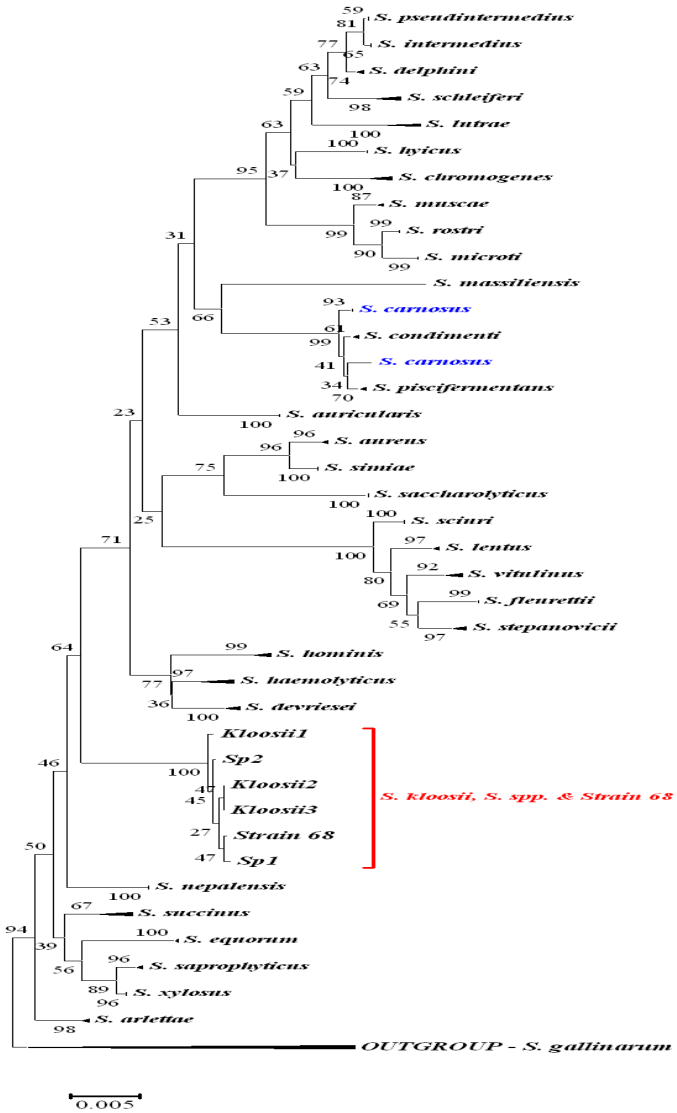

Figure 2: Evolutionary relationships of *Staphylococcus* species using partial 16S ribosomal RNA gene sequences.
Note: Blue, non-monophyletic cluster; red, focal cluster

Table 1: Taxa incorporated in the phylogenetic analyses of *Staphylococcus* species using partial 16S ribosomal RNA gene sequences.

Taxa	Sample size	Individual no.	GenBank accession no.
Staphylococcus sp.	1	Strain 68	JX102547
Staphylococcus sp.	1	Sp.1	GU451172
Staphylococcus sp.	1	Sp.2	HM352369
S. arlettae	3	Arlettae1	HQ154573
		Arlettae2	HQ154557
		Arlettae3	NR024664
S. aureus	3	Aureus1	AB681717
		Aureus2	AB681715
		Aureus3	AB681713
S. auricularis	3	Auricular1	NR036897
		Auricular2	D83358
S. carnosus	3	Carnosus1	EU727181
		Carnosus2	EU727182
		Carnosus3	NR027518
S. chromogenes	3	Chromogen1	JN426805
		Chromogen2	NR036901
		Chromogen3	D83360
S. condimenti	3	Condimen1	EU727183
		Condimen2	NR029345
		Condimen3	Y15750
S. delphini	3	Delphini1	NR024666
		Delphini2	AB009938
		Delphini3	HQ452512
S. devriesei	3	Devriesei1	FJ938168
		Devriesei2	FJ389207
		Devriesei3	FJ389208
S. equorum	3	Equorum1	NR027520
		Equorum2	EU221367
		Equorum3	EU221366
S. fleurettii	2	Fleuretti1	NR041326
		Fleuretti2	AB233330

(*continued on next page*)

Table 1: (*continued*)

Taxa	Sample size	Individual no.	GenBank accession no.
S. gallinarum	3	Gallinaru1	NR036903
		Gallinaru2	DQ350835
		Gallinaru3	FN646072
S. haemolyticus	3	Haemolyti1	NR036955
		Haemolyti2	EU554432
		Haemolyti3	JN644560
S. hominis	3	Hominis1	JQ677135
		Hominis2	JQ734768
		Hominis3	NR036956
S. hyicus	2	Hyicus1	NR036905
		Hyicus2	D83368
S. intermedius	3	Intermedi1	AB626130
		Intermedi2	NR036829
		Intermedi3	D83369
S. kloosii	3	Kloosii1	JQ660048
		Kloosii2	JQ660231
		Kloosii3	JQ660155
S. lentus	3	Lentus1	JN673760
		Lentus2	NR043418
		Lentus3	AY395014
S. lutrae	2	Lutrae1	NR036791
		Lutrae2	AB233333
S. massiliensis	1	Massilien1	EU707796
S. microti	2	Microti1	EU888120
		Microti2	EU888123
S. muscae	2	Muscae1	FR733703
		Muscae2	S83566
S. nepalensis	3	Nepalensi1	AB697721
		Nepalensi2	AB697719
		Nepalensi3	AB697720
S. piscifermentans	3	Pisciferm1	NR036981
		Pisciferm2	EU727184
		Pisciferm3	Y15754

(*continued on next page*)

Table 1: (*continued*)

Taxa	Sample size	Individual no.	GenBank accession no.
S. pseudintermedius	3	Pseudinte1	NR042284
		Pseudinte2	GU057859
		Pseudinte3	GU057858
S. rostri	2	Rostri1	FM242137
		Rostri2	AM989462
S. saccharolyticus	3	Saccharol1	AB646616
		Saccharol2	NR029158
		Saccharol3	L37602
S. saprophyticus	3	Saprophyt1	AB681788
		Saprophyt2	JQ229688
		Saprophyt3	JQ309134
S. schleiferi	3	Schleifer1	JQ407790
		Schleifer2	NR037009
		Schleifer3	AB009945
S. sciuri	3	Sciuri1	HQ154580
		Sciuri2	HQ154558
		Sciuri3	NR025520
S. simiae	3	Simiae1	NR043146
		Simiae2	AY727530
		Simiae3	DQ127902
S. stepanovicii	3	Stepanovi1	GQ222245
		Stepanovi2	GQ222243
		Stepanovi3	GQ222244
S. succinus	3	Succinus1	HQ018602
		Succinus2	JF920302
		Succinus3	JN644525
S. vitulinus	3	Vitulinus1	NR024670
		Vitulinus2	AM062694
		Vitulinus3	AB009946
S. xylosus	3	Xylosus1	AB626129
		Xylosus2	JN644524
		Xylosus3	NR036907

Table 2: Estimates of evolutionary divergence between partial 16S ribosomal RNA gene sequences of bacterial Strain 68 and other members of *S. kloosii, Staphylococcus spp.* and the Strain 68 cluster.

Sample	Strain 68	Sp.1	Sp.2	Kloosi1	Kloosi2	Kloosi3
Strain 68	–	0.001	0.001	0.001	0.001	0.001
Sp.1	0.001	–	0.001	0.001	0.001	0.001
Sp.2	0.002	0.002	–	0.001	0.001	0.001
Kloosii1	0.002	0.002	0.002	–	0.001	0.001
Kloosii2	0.001	0.002	0.001	0.001	–	0.000
Kloosii3	0.001	0.002	0.001	0.001	0.000	–

Table 3: Percentage (%) nucleotide composition of unaligned partial 16S ribosomal RNA gene sequences of bacterial Strain 68 and other members of *S. kloosii, Staphylococcus spp.* and the Strain 68 cluster.

Sample	Thymine (T)	Cytosine (C)	Adenine (A)	Guanine (G)	Total nucleotide bases
Strain 68	21.6	22.6	26.7	29.1	1365
Sp.1	21.7	22.5	26.7	29.1	1366
Sp.2	21.6	22.6	26.7	29.1	1365
Kloosii1	21.5	22.6	26.9	29.0	1366
Kloosii2	21.5	22.6	26.8	29.1	1366
Kloosii3	21.6	22.6	26.8	29.1	1368

Pigment Extraction, Wavelength Scan and Growth Curve Determination

A single bacterial colony of Strain 68 that was phylogenetically identified as *S. kloosii* was inoculated into 200 ml of nutrient broth media (NB) [CM0001, Oxoid, UK; ingredients: 'Lab-Lemco' powder, 1 g/l; yeast extract, 2 g/l; peptone, 5 g/l; sodium chloride, 5 g/l] in a 1 litre Erlenmeyer flask and incubated at 37°C with shaking at 150 rpm until the inoculum had grown well and produced orange pigment that made the NB turbid.

The pigmented culture was transferred into a Falcon™ (USA) 50 ml conical tube and centrifuged at 9000 rpm for 10 min at 18°C using a Hettich Universal (Tuttlingen, Germany) 320R centrifuge. The supernatant obtained after centrifugation was discarded, and the pellets containing intracellular pigments were mixed with 5 ml of absolute methanol in a Falcon™ 50 ml conical tube. Methanol and ethanol were used as single extracting solvents. The mixture of bacterial cells and 5 ml of absolute solvent was vortexed to mix well, and the conical tube was incubated at 37°C with shaking at 150 rpm for up to 2 h to extract the intracellular pigments from the bacterial cells. After the extraction, the mixture was centrifuged at 9000 rpm for 10 min at 18°C. For the chromatographic analysis, the pigment extracts were concentrated from 5 ml to approximately 250 µl using a GeneVac miVac DNA Sample Concentrator (Ipswich, United Kingdom) with a volatile solvent option. A wavelength scan was performed from

390 to 560 nm using a PerkinElmer LAMBDA 35 UV/Vis (Massachusetts, USA) spectrophotometer to determine the visible spectra of ethanolic and methanolic pigment extracts from bacterial Strain 68 (Fig. 3). A cell growth curve of bacterial Strain 68 was determined by measuring the optical density at 600 nm (OD_{600}) of 1 ml of NB containing Strain 68 once per hour (Fig. 4). Pigment production was also observed and measured at the visible wavelengths derived from the wavelength scan (the curve is not shown in Fig. 4).

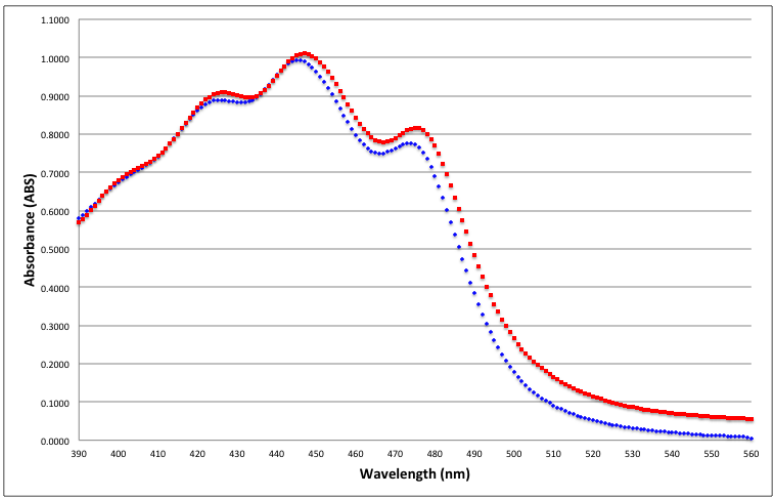

Figure 3: Visible spectra of ethanolic (red spectrum, λ_{max} at 426, 447 and 475 nm) and methanolic (blue spectrum, λ_{max} at 426, 445 and 473 nm) pigment extracts of bacterial Strain 68.

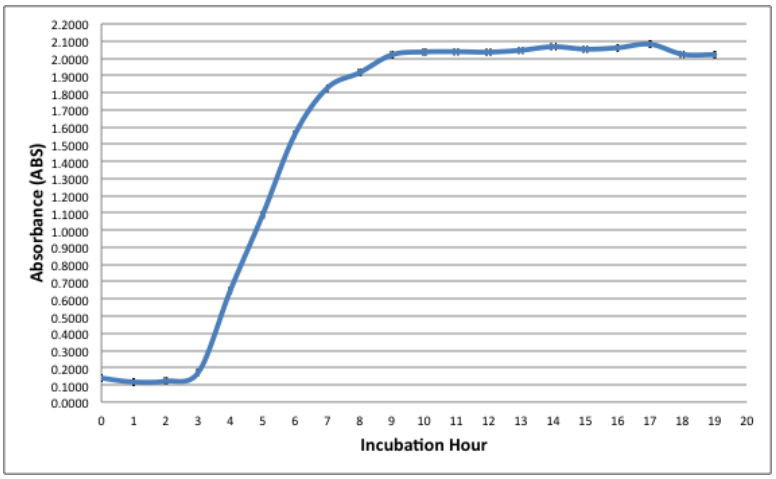

Figure 4: Cell growth of bacterial Strain 68. The exponential phase of cell growth was reached between 2.5 and 9 hours of incubation. Pigment production began between hour 5 and hour 6 of incubation and reached its highest level at hour 16 (the curve is not shown).

TLC Analysis

A Merck Millipore (Darmstadt, Germany) classical TLC silica gel 60 F_{254} plate was cut into small plates 10 cm in length x 2 cm in width. A small amount of methanolic pigment extract from Strain 68 was centrally spotted 1 cm from the bottom of the small TLC plate (i.e., the start line/starting point), and a hair dryer was used to dry the methanol solvent. A chloroform:methanol (30:0.5) TLC solvent was prepared and placed in a glass chamber. The atmosphere in the chamber was equilibrated with the TLC solvent for 5 min. The dried TLC plate was placed in the chamber, and the solvent front was allowed to run up the plate. When the solvent front had run far enough, the TLC plate was removed from the TLC chamber. The TLC plate was dried, and the pigments were visualised using a Spectroline Model CM-10 (Westbury, New York) fluorescence analysis cabinet and iodine staining. The retardation factor (R_f) of each pigment compound or coloured fraction spot observed on the TLC plate was measured using the formula $R_f = a/b$ (a = the distance in cm from the starting point to the centre of the spot on the TLC plate, and b = the distance in cm from the starting point to the solvent front).

RESULTS AND DISCUSSION

DNA sequencing of the 16S ribosomal RNA gene showed the presence of *S. kloosii* Strain 68 in the respiratory tree of *H. leucospilota* (Brandt 1835) from Teluk Nipah, Pangkor Island, Perak, Malaysia. This report is the first documentation of the isolation of an *S. kloosii* strain in Malaysia, as no record could be found previously. The bacterial colonies were orange on TGYEA with a pH of 7.19. This bacterial strain is a mesophile because it grew well at 37°C, i.e., human body temperature. The specimens of *H. leucospilota* sampled in Teluk Nipah, Pangkor Island inhabited seawater at a temperature of 29.17°C (i.e., ambient temperature) with a pH between 6 and 7, which agrees with the laboratory observations. The bacterial strain is Gram-positive due to its violet or purple colour resulting from Gram staining (Fig. 1), which confirms that it is a *Staphylococcus* species. A grape-like clustering arrangement, which is another characteristic of *Staphylococcus*, is not obvious in Figure 1 as the cells were too crowded. The bacterial shape is spherical or coccus (Fig. 1) with a circular form, raised elevation, continuous margin, smooth surface, opacity and chromogenesis, i.e., orange pigmentation as observed under the dissecting microscope and by the naked eye. Its typical growth pattern in broth media (i.e., NB) was uniformly turbid, and the cells were diffused throughout.

The partial 16S rRNA gene sequence of bacterial Strain 68 was registered with GenBank, (Table 1, GenBank accession no. JX102547; retrieved at http://www.ncbi.nlm.nih.gov/nuccore/JX102547). A total of 96 partial 16S ribosomal RNA gene sequences from 34 known and 2 unknown species of genus *Staphylococcus* were obtained from GenBank for the phylogenetic analyses of *Staphylococcus* species. In total, 97 partial 16S ribosomal RNA gene sequences were included (Table 1). The neighbour-joining method (Fig. 2) grouped Strain 68 with all the DNA sequences of *S. kloosii* from GenBank with a 100% bootstrap

value, suggesting that Strain 68 is *S. kloosii*. The number of base substitutions per site between sequences of Strain 68 and *S. kloosii* are few (Table 2), thus supporting the results shown in the neighbour-joining tree. More interestingly, both of the unknown *Staphylococcus* species from GenBank, i.e., Sp.1 (GenBank accession no. GU451172) and Sp.2 (GenBank accession no. HM352369), are thought to be *S. kloosii* because the average level of evolutionary divergence between them and the other *S. kloosii* cluster members (Fig. 2) is very low (Table 2). Thus, the total number of *Staphylococcus* species incorporated in this study can be increased to 34. Based on the data from the neighbour-joining tree and evolutionary divergence, the nucleotide composition of unaligned partial 16S ribosomal RNA gene sequences of Strain 68 and other members of *S. kloosii, Staphylococcus spp.* and the Strain 68 cluster are considered identical, despite the very low percentage difference (Table 3).

Pigments from *S. kloosii* bacterial Strain 68 were found to be intracellular based on their orange pellets and the fact that the NB was not turbid after centrifugation. A pigment is considered extracellular if, after centrifugation, the NB is still turbid or the colour of the NB (which changes during fermentation due to pigment synthesis) remains the same. In fact, colour hue is dependent on pigment concentration; yellow pigments gradually turn orange and even red at increasing concentrations. Therefore, the determination of the colour hue of bacterial Strain 68 pigment is subjective. Strain 68 pigment was also soluble in ethanol and methanol. The wavelength scan ranged from 390 to 560 nm, and the visible spectra of ethanolic (λ_{max} at 426, 447 and 475 nm) and methanolic (λ_{max} at 426, 445 and 473 nm) pigment extracts of bacterial Strain 68 were considered identical (Fig. 3). Both visible spectra likely resembled the visible spectra of lutein (Rodriguez-Amaya & Kimura 2004; Zang *et al*. 1997). According to Sommerburg *et al*. (1998), eating green leafy vegetables enriched with lutein and zeaxanthin may help to decrease the risk of age-related macular degeneration, which is a major cause of blindness and visual impairment. Compared with lutein production from plant materials, lutein production via microbial fermentation has a number of advantages including (1) cheaper production, (2) potentially increased ease of extraction, (3) higher yields (especially through strain improvement), (4) no lack of raw materials and (5) no seasonal variations (Mortensen 2006). However, additional research is needed to further identify the pigment compounds in bacterial Strain 68.

Figure 4 shows that the lag phase occurred before hour 3 for approximately 3 hours, and the exponential phase of Strain 68 cell growth was reached between 2.5 and 9 hours of incubation. Furthermore, the stationary phase began at hour 9. Regarding pigment biosynthesis, the pigment production of Strain 68 started during the exponential phase, between hour 5 and hour 6 of incubation, and reached the highest level during the stationary phase at hour 16 (the curve is not shown). The determination of the bacterial growth curve and knowledge regarding pigment production are very important when scaling up microbial pigment production for industrial purposes.

Fractionation of a methanolic extract of bacterial Strain 68 pigment by TLC indicated at least three visible fractions or spots (Fig. 5): an orange pigment compound (the major compound), a yellow pigment compound (the least polar

compound), and a pink pigment compound (the most polar compound). The colourless ultraviolet-active organic compounds visible under long wavelength UV light and by staining the TLC plate with iodine vapour suggest that pure orange pigment compound (R_f = 0.6) and pure pink pigment compound (R_f = 0.2) can be obtained using gravity column chromatography; however, the yellow pigment compound, which was the least polar compound (R_f = 1.0), requires a few more purification steps including gravity column chromatography because the compound is mixed with one or more colourless ultraviolet-active organic compounds (Fig. 5). In general, the ideal R_f range is $0.2 \leq R_f \leq 0.8$. These findings are considered to be the first documentation of the pigment composition of an *S. kloosii* strain, as no such record could be found prior to now.

In the future, purification and identification of the structures and characteristics of the isolated *S. kloosii* pigment compounds using common techniques such as nuclear magnetic resonance (NMR), mass spectrometry (MS), UV-Vis spectroscopy and infrared (IR) spectroscopy will be essential for determining the potential utility of these pigment compounds as food-grade microbial pigments in the natural food colourant industry.

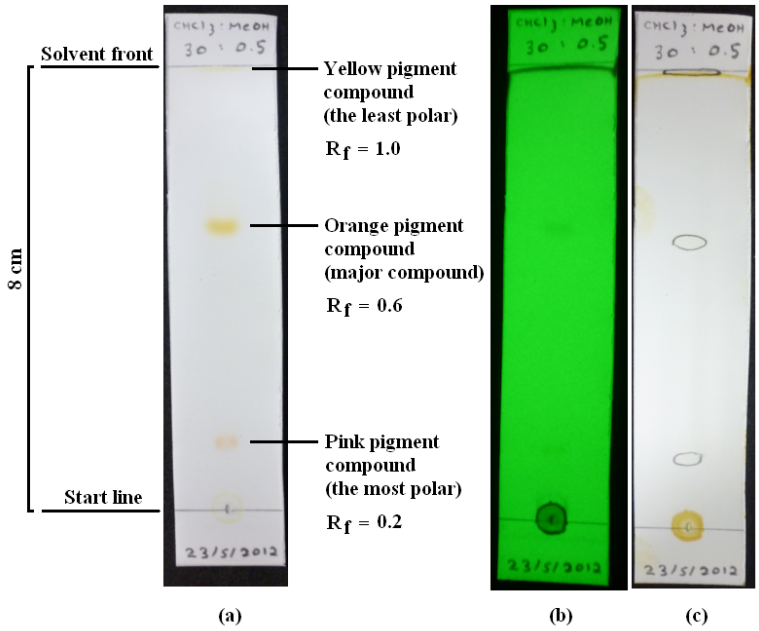

Figure 5: Fractionation of a methanolic extract of bacterial Strain 68 pigment by TLC: (a) a methanolic extract of Strain 68 pigment was fractionated on a silica-coated TLC plate using chloroform:methanol (30:0.5), and at least three fractions were visibly obtained: an orange pigment compound (major compound), a yellow pigment compound (the least polar compound), and a pink pigment compound (the most polar); (b) colourless ultraviolet-active organic compounds visible under long wavelength UV light; (c) colourless organic compounds visible by staining the TLC plate with iodine vapour.

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