ORIGINAL ARTICLE

Identification and biodegradation characteristics of oil-degrading bacteria from subtropical Iriomote Island, Japan, and tropical Con Dao Island, Vietnam

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ABSTRACT Indigenous oil-degrading bacteria (ODB) were screened from two islands, Iriomote, Japan and Con Dao, Vietnam. These islands are considered predicted contamination sites from oil spillage on a nearby busy transportation route. The aim of this study was to compare the degradation abilities of bacterial isolates from the two different study areas for crude oil, *n*-alkanes, and polycyclic aromatic hydrocarbons. Comparing the two study areas, the numbers of the total bacteria and ODB showed the same distribution tendencies. There were no differences between Iriomote and Con Dao in the counts of the total bacteria and ODB in sediment samples, while those in seawater samples were statistically lower for Iriomote than for Con Dao. A total of 45 isolates, 25 from Iriomote and 20 from Con Dao, belonging to 18 genera, were isolated. Among 11 genera detected on Iriomote, the dominant genera were *Achromobacter*, *Pseudomonas*, and *Ochrobactrum*. Among 11 genera from Con Dao, the dominant genera were *Pseudomonas* and *Microbacterium*. The degradation ability of the isolates was studied for crude oil, two kinds of *n*-alkanes, and two kinds of polycyclic aromatic hydrocarbons (PAHs). The numbers of the isolates that degraded more than 50 percent of the crude oil and *n*-alkanes and 25 percent of the PAHs present in the media were higher for Iriomote (3, 11, and 2, respectively) than for Con Dao (2, 4, and 1, respectively). It is noteworthy that the isolates of *Pseudomonas putida* and *Ochrobactrum anthropi* could degrade more than 40 percent of the oil and *n*-alkanes and more than 25 percent of the PAHs present in the media.

Key words: Autochthonous bioaugmentation, Biodegradation, Bioremediation, Oil-degrading bacteria, Oil spill treatment

INTRODUCTION

Oil spillage from waterborne transportation is considered a significant pollution source, which accounts for four million barrels of crude oil discharged through seepage per year and for 70 percent of pollution due to oil and gas activities (Weis 2015). Marine and coastal areas are seriously affected and exposed to enormous environmental damage caused by oil spillage.

Bioremediation is a biological method, which is considered an effective technique for treating oil contamination. In recent years, a new concept, called autochthonous bioaugmentation, has been introduced that promises the most effective remediation method for oil contamination. In this method, indigenous microorganisms are used, which

are well characterized as oil degraders from contaminated sites or predicted contamination sites. This method showed a number of advantages for remediating oil-polluted land, with shorter treatment times, a greater potential efficiency, lower impact on the environment, and easier public acceptance (Hosokawa et al. 2009).

In this study, we selected two islands, which are considered predicted contamination areas because of oil spillage on a nearby busy transportation route. One is the subtropical Iriomote Island, the largest of the Yaeyama Islands located in the southernmost region of Japan in the East China Sea. The other is the tropical Con Dao Island located southeast of Vietnam in the South China Sea. These islands are designated as national parks, 70-80 percent of which are covered with sensitive ecosystems, including

jungles, mangroves, and coral reefs, and inhabited by several endangered species. However, the islands are located on a busy maritime route, by which 15 million barrels of oil are transported per day. This route supplies 83 percent of oil and oil products to Japan and East Asian countries (EIA 2014). In addition, the Con Dao Island is located on the biggest oil fields in Vietnam.

We previously compared the oil-degrading fungal distributions between the two different study areas, subtropical Iriomote Island and tropical Con Dao Island (Doan et al. 2016). The aim of this study was to compare the degradation abilities of bacterial isolates from two different study areas, subtropical Iriomote Island and tropical Con Dao Island, for crude oil, *n*-alkanes, and polycyclic aromatic hydrocarbons (PAHs). We isolated and screened indigenous oil-degrading bacteria (ODB) from both islands. Because oil is a complex mixture of hydrocarbons and no single species can completely degrade all components (Chikere et al. 2011), degradation of two alkanes, heptadecane and octadecane,

and two PAHs, fluoranthene and pyrene, was representatively used. Furthermore, potential pathogenicity of the isolates to other living organisms was assessed to compare isolates from the two islands.

MATERIALS AND METHODS

Study areas and sample collection

Total bacteria and ODB were enumerated and isolated from sediment (S) samples and seawater (W) samples from the two islands, Iriomote and Con Dao (Fig. 1), as in the previous study (Doan et al., 2016). Four study sites were designed on each island, including the Urauchi mangrove forest (Ir1), Uehara Port (Ir2), Funaura Bay (Ir3), and Ohara Port (Ir4) on Iriomote and the Dam Trau Beach (CD1), Vong Beach (CD2), An Hai Port (CD3), and Ben Dam Port (CD4) on Con Dao (Table 1).

Fig. 1. Locations of the study areas on the Iriomote and Con Dao islands along the transportation route through the Strait of Malacca.

The coordinates of the sampling sites are shown in Table 1. In each site, two S samples, from supratidal and intertidal zones, and one W sample were taken during the dry season, from January to May 2014. Each sample was collected from four random locations in each sampling site and transferred to the laboratory in sterilized glass bottles in a cool box at 4–8°C to be analyzed within 48 h. Environmental parameters, pH, salinity of seawater, and air temperature were determined in each sampling site.

Medium preparation

For culturing total bacteria and ODB, tryptic soy agar (TSA; Wako Ltd., Japan) and mineral salt agar (MSA) were used, respectively (Chaillan et al. 2004). MSA consisted of KH_2PO_4 , 0.68 g; Na_2HPO_4 , 1.79 g; MgSO_4 , 0.35 g; NH_4NO_3 , 1 g; CaCl_2 , 0.02 g; FeSO_4 , 0.4 mg; and agar, 15 g in 1 l of distilled water and was supplemented with 0.1 ml of a solution containing 100 mg/l of each element H_3BO_3 , MnSO_4 , ZnSO_4 , CuSO_4 , and CoCl_2 . The medium was supplemented with 1 percent (v/v) of weathered Seria Light Export Blend crude oil supplied by the Brunei Shell Petroleum Company, Brunei Darussalam, as a sole carbon source. The oil was weathered by heating at 100°C for 48 h, sterilized by membrane filters (47 mm diameter, 0.2 µm pore size), and spread on the surface of MSA plates (Venkateswaran 1991). Cycloheximide (0.1 g/l) was aseptically added as an antifungal agent to the media after sterilization and cooling to

50°C.

For ODB enrichment and degradation experiments, mineral salt broth (MSB) was used, which had the same composition as MSA, except agar. The weathered oil (10 ml/l) and cycloheximide (0.1 g/l) were added to the sterilized medium as a sole carbon source and an antifungal agent, respectively (Venkateswaran 1991).

Enumeration of total bacteria and ODB

The S samples were treated aseptically. They were weighed and homogenized in a 50 percent (w/v) sodium chloride solution (0.85 percent, w/v), and the adhered microorganisms were dissociated by vortexing. A decimal serial dilution of a sample was spread on TSA and MSA plates for the enumeration of total bacteria and ODB, respectively. Each dilution was inoculated in triplicate. The grown colonies were counted, and the results were expressed as colony-forming units (CFU) per gram of dry weight of an S sample.

Bacteria in the W samples were enumerated by the membrane filter method (Venkateswaran 1991). A volume of 100 ml of a W sample was filtered through a membrane (47 mm diameter, 0.2 µm pore size) in duplicate. One membrane was placed on the top of a TSA plate, and the other was placed on the top of an MSA plate for the enumeration of total bacteria and ODB, respectively. The developed colonies were counted, and the results were

Table 1. Coordinates and environmental parameters of sampling sites on the Iriomote and Con Dao islands

Sampling sites	Abbreviation	Coordinates	pH	Salinity (%)	Air temperature (°C)
Iriomote					
Urauchi mangrove forest	Ir1	24.4042 N 123.7774 E	7.9	2.5	15.3
Uehara Port	Ir2	24.4142 N 123.8030 E	8.1	3.5	15.1
Funaura Bay	Ir3	24.4007 N 123.8117 E	8.0	3.3	16.4
Ohara Port	Ir4	24.2757 N 123.8820 E	8.0	3.2	16.8
Con Dao					
Dam Trau Beach	CD1	08.7328 N 106.6220 E	8.2	3.5	34.2
Vong Beach	CD2	08.7130 N 106.6382 E	8.1	3.4	34.8
An Hai Port	CD3	08.6813 N 106.6088 E	8.1	3.5	35.1
Ben Dam Port	CD4	08.6603 N 106.5698 E	8.1	3.5	35.5

expressed as CFU per 100 ml of a W sample. All incubations were carried out at 25°C up to 7–10 d.

Isolation of ODB

ODB were directly isolated by picking up the colonies grown on the MSA medium in the enumeration step and repeatedly streaked on new TSA plates. ODB were also isolated by the enrichment medium method (Chaerun et al. 2004) described below. One gram of an S sample was inoculated in an Erlenmeyer flask containing 50 ml of MSB medium, and the flask was incubated at 120 rpm, 25°C for 5–20 d. After every 5 d of incubation, the culture was diluted, and an aliquot was spread on MSA medium. The formed colonies were isolated and repeatedly streaked on new TSA plates.

Identification of isolates

The generic identities of the isolates were determined biochemically using API kits (bioMérieux, Japan) following the manufacturer's protocols. The API kits contained dehydrated substrates in microtubes to detect enzymatic activities for the assimilation or fermentation of sugars.

The species identification was performed by a molecular method using 16 S ribosomal ribonucleic acid (16 S rRNA) gene sequences. The isolates were cultured on TSA slants at 25°C for 2 d, and DNA was extracted by the alkaline wash/lysis method (Millar et al. 2000). The 16 S rRNA gene was amplified by polymerase chain reaction (PCR) using the bacterial universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') (Chaerun et al. 2004). PCR reactions were performed in a PCR Thermal Cycler MP (TaKaRa) (Tokuda et al. 2000). The PCR samples were directly sequenced on an ABI PRISM 3100 sequencer (Applied Biosystems, Foster City, CA, USA) using the primers 27f and 1492r. The sequences were aligned using the ATGC Ver.7 Sequence Assembly software for Windows (GENETYX Corporation) and MEGA Ver. 6 (Tamura et al. 2013). The sequences were analyzed by the National Center for Biotechnology Information BLAST tool to identify closely related bacterial sequences. The isolates were identified to a certain species if the BLAST results showed similarity values of higher than 97 percent.

Screening for oil degradation

Oil degradation abilities of the isolates were verified using a modified technique based on the redox indicator *p*-iodonitrotetrazolium (INT) (Wrenn and Venosa 1996). The isolates were cultivated in 5 ml of MSB supplemented with oil. After 7 d, 100 µl of filter-sterilized INT (5 g/l) was added, and the color change of the mixture was observed after overnight incubation. The color change of INT from light yellow to pink or red was scored as + or ++, respectively. The darker medium color indicated better bacterial growth in the medium with oil.

Temperature effect on the isolates

Temperature resistance is a characteristic that shows bioremediation application and pathogenic potentials of isolates. Our isolates were cultured on TSA slants in duplicate at temperatures of 15, 20, 30, 35, 37, 39, and 42°C, and their growth was recorded after 7 d. After the incubation, the test tubes with no growth were kept at 25°C for 7 d to check for bacterial survival.

Biodegradation of crude oil and *n*-alkanes

The degradation of oil and the *n*-alkanes, heptadecane and octadecane, by the isolates was determined by the gravimetric method (Chaillan et al. 2004). The isolates were grown in duplicate without shaking in 250-ml Erlenmeyer flasks containing 100 ml of MSB medium and 500 mg of oil or 100 mg of each *n*-alkane as the sole carbon source. Sterile controls were used to quantify abiotic losses due to evaporation. After 30 d of incubation at 28 ± 2°C, microbial activities were stopped by adding 1 ml of 1 N HCl to the flasks. The residual oil was recovered by dichloromethane extraction (Chaillan et al. 2004). The degradation rate was determined as $B(\%) = 100 \times (W_1 - W_c)/W_1$, where W_1 is the residual mass in the control and W_c is that in the culture.

Biodegradation of PAHs

The two PAHs, fluoranthene and pyrene, were used for determining the degradation abilities of the isolates. A solution of fluoranthene or pyrene in *N,N*-dimethyl-formamide was added at a final concentration of 100 mg/l to 100 ml of MSB medium in 250-ml Erlenmeyer flasks. The incubation and solvent extraction were carried out

under the same conditions as in the oil degradation experiments described above. After the extraction, residual PAH concentrations were determined by a gas chromatography-mass spectrometry method (Mineki et al. 2015). The degradation rate was calculated using the same formula as described above for the oil degradation rate.

Statistical analysis

The data were analyzed using Microsoft Excel. Differences in the total bacteria or ODB counts between Iriomote and Con Dao were analyzed by the Student's *t*-test ($p < 0.05$). Differences in the total bacteria or ODB counts among the four study sites were analyzed by analysis of variance ($p < 0.05$).

RESULTS

Environmental parameters of sampling sites

The environmental parameters of the sampling sites are shown in Table 1. The pH and salinity values were not much different among all sampling sites on Iriomote and

Con Dao, except Ir1. This site had the lowest values of pH (7.9) and salinity (2.5 percent). The air temperatures ranged between 15.1 and 16.8°C on Iriomote and 34.2 and 35.5°C on Con Dao.

Distribution of total bacteria

Comparing the average numbers of total bacterial counts obtained from the S and W samples taken on Iriomote and Con Dao, there were no differences in the results for the S samples, whereas the numbers for the W samples taken on Con Dao were statistically larger than those for the W samples taken on Iriomote. The bacterial count distributions for the sampling sites are shown in Fig. 2. On Iriomote, the total numbers of bacteria ranged from 6.5×10^4 (Ir3) to 5.7×10^5 CFU g⁻¹ (Ir1) in the S samples and from 3.3×10^2 (Ir3) to 1.2×10^3 CFU 100 ml⁻¹ (Ir4) in the W samples. The total numbers of bacteria on Con Dao ranged from 2.3×10^4 (CD1) to 2.4×10^6 CFU g⁻¹ (CD4) in the S samples and 4.0×10^3 (CD1 and CD3) to 6.3×10^3 CFU 100 ml⁻¹ (CD4) in the W samples. On Iriomote, Ir1 gave the highest total number of bacteria among the S samples, whereas Ir4 gave the highest total number of bacteria among the W samples. On Con Dao, CD4 gave the

Fig. 2. The counts of the total bacteria and oil-degrading bacteria (ODB) in the sediment (S) and seawater (W) samples from the four sites on the Iriomote and Con Dao islands.

Ir1-4 and CD1-4 are shown in Table 1.

The bars represent standard deviations.

The asterisks represent the statistically highest numbers of total bacteria or ODB in the S and W samples from the four sites on Iriomote and Con Dao as determined by analysis of variance ($p < 0.05$).

highest total number of bacteria among both S and W samples.

Distribution of ODB

Comparing the two study areas, the numbers of ODB showed the same distribution tendency as the total numbers of bacteria. On Iriomote, the numbers of ODB in the S samples ranged from 1.6×10^4 (Ir2) to 2.7×10^4 CFU g⁻¹ (Ir1), and those in the W samples ranged from 6.8×10^1 (Ir3) to 1.6×10^2 CFU 100 ml⁻¹ (Ir4). The numbers of ODB in the S samples from Con Dao ranged from 3.1×10^2 (CD1) to 1.7×10^5 CFU g⁻¹ (CD4), and those in the W samples ranged from 3.2×10^2 (CD1) to 1.1×10^3 CFU 100 ml⁻¹ (CD4). On Iriomote, Ir4 was characterized by the highest number of ODB in the W samples among the four sites. On Con Dao, CD4 gave significantly higher numbers of ODB for the S and W samples. On Iriomote, the highest percentages of ODB in the total bacterial population were found at Ir3, 27.1 percent in the S samples and 20.5 percent in the W samples. The highest percentages of ODB in the total bacterial population on Con Dao were obtained at CD3, 17.4 percent in the S samples and 19.4 percent in the W samples.

Isolation and identification of ODB

A total of 45 ODB, which could grow in the medium with oil, were isolated from Iriomote and Con Dao. The 25 isolates from Iriomote were identified as belonging to 11 genera, *Achromobacter* (five isolates), *Acinetobacter* (three isolates), *Alcaligenes* (one isolate), *Burkholderia* (two isolates), *Dietzia* (one isolate), *Gemella* (one isolate), *Ochrobactrum* (four isolates), *Paenibacillus* (one isolate), *Pseudomonas* (five isolates), *Vibrio* (one isolate), and a Gram-negative coccus (one isolate). The 20 isolates from Con Dao were identified as members of 11 genera, *Achromobacter* (one isolate), *Brachybacterium* (one isolate), *Gemella* (two isolates), *Granulicatella* (one isolate), *Halomonas* (one isolate), *Klebsiella* (two isolates), *Microbacterium* (three isolates), *Ochrobactrum* (one isolate), *Pseudomonas* (six isolates), *Yersinia* (one isolate), and a Gram-positive bacillus (one isolate). The sequences of the isolates were submitted to the genetic sequence database at DDBJ/EMBL/GenBank with the accession numbers shown in Table 2.

Oil degradation by the isolates

A total of 45 isolates had the ability to grow in the medium with oil as the sole carbon source (Table 2). The ability of the isolates to degrade oil was rated at two levels with INT. On Iriomote, 10 isolates, Oh-1.13 from the genus *Achromobacter*, Ue2-1.1 and Ur-2.10.2 from *Acinetobacter*, Oh-1.14 from *Burkholderia*, Fu2-1.4 from *Paenibacillus*, Oh2-1.3, Ue2-1.2, Ue2-2.5.2, and Oh-1.15 from *Pseudomonas*, and Fu2-2.1 from *Vibrio* showed high abilities to degrade oil (+ +). On Con Dao, nine isolates, VB-2.1.2 from the genus *Brachybacterium*, DT-2.15.1 from *Gemella*, DT-2.3.1 from *Granulicatella*, VB-1.1 from *Klebsiella*, BD-2.4.2 from *Microbacterium*, BD-2.5.3 from *Ochrobactrum*, BD-1.5.1 and BD-2.5.6 from *Pseudomonas*, and DT-1.15.2 from *Yersinia* showed high abilities to degrade oil (+ +).

Temperature effect and pathogenic potential

The growth and survival of the isolates at the temperatures from 15 to 42°C are shown in Table 2. In the range from 15 to 35°C, there was no temperature effect on the growth of the isolates. Among the Iriomote isolates, one isolate, Oh-1.0.2, could grow and survive only up to 35°C. Two isolates, Fu-3.1.1 and Oh-1.15.2, grew up to 37 and survived at 42°C, and one isolate, Ir-2, grew up to 39 and survived at 42°C. Among the Con Dao isolates, one isolate, AH-1.15.1, grew and survived only up to 35°C and 37°C, respectively. Isolate VB-1.14.2 grew up to 37 and survived at 42°C, and two isolates, VB-1.11 and BD-2.4.2, grew up to 39 and survived at 42°C.

Based on the results of the species identification and the temperature effects (Govan et al. 1996, Podschun and Ullmann, 1998 and Cargill et al. 2012), one isolate, *Burkholderia cepacia* Oh-1.14, from Iriomote and three isolates, *Granulicatella adiacens* DT-2.3.1, *Klebsiella pneumoniae* VB-1.1, and *K. pneumoniae* VB-1.5, from Con Dao were determined as potentially pathogenic. We assigned numbers to the rest of the isolates and used them for further studies (Table 2).

Biodegradation rates of crude oil by the isolates

The results of the oil degradation rates shown by the isolates are presented in Fig. 3. Among the Iriomote isolates, three isolates, No. 6 (Ue2-1.1, 71.2 percent), No. 21 (Ue2-2.5.2, 53.0 percent), and No. 23 (Oh-1.15, 55.1

Table 2. List of bacterial isolates from the Iriomote and Con Dao islands and their characteristics.

No. ¹⁾	Isolate	Species	Accession No.	Oil-degradation screening ²⁾	Maximum growth temperature ³⁾ (°C)	Maximum survival temperature ³⁾ (°C)
Iriomote						
1	Ue-1.2	<i>Achromobacter denitrificans</i>	LC125138	+	42	42
2	Ue-2.2	<i>Achromobacter xylosoxidans</i>	LC125142	+	42	42
3	Oh-1.13	<i>Achromobacter</i> sp.	LC125139	++	42	42
4	Ir-1	<i>Achromobacter</i> sp.	LC125140	+	42	42
5	Ir-12.2	<i>Achromobacter</i> sp.	LC125141	+	42	42
6	Ue2-1.1	<i>Acinetobacter</i> sp.	LC125143	++	42	42
7	Oh-2.10.0	<i>Acinetobacter</i> sp.	LC125144	+	42	42
8	Ur-2.10.2	<i>Acinetobacter</i> sp.	LC125145	++	42	42
9	Fu-2.2	<i>Alcaligenes</i> sp.	LC125146	+	42	42
–	Oh-1.14	<i>Burkholderia cepacia</i>	– ⁴⁾	++	42	42
10	Ir-5	<i>Burkholderia</i> sp.	–	+	42	42
11	Fu2-3.2	<i>Dietzia</i> sp.	LC125147	+	42	42
12	Ur-2.5.1	<i>Gemella</i> sp.	–	+	42	42
13	Oh-1.14.2	Gram – ve cocci	–	+	42	42
14	Ue-1.1	<i>Ochrobactrum anthropi</i>	LC125148	+	42	42
15	Fu-3.1.1	<i>Ochrobactrum anthropi</i>	–	+	37	42
16	Ir-2	<i>Ochrobactrum anthropi</i>	LC125149	+	39	42
17	Oh-1.15.2	<i>Ochrobactrum</i> sp.	LC125150	+	37	42
18	Fu2-1.4	<i>Paenibacillus</i> sp.	LC125151	++	42	42
19	Oh2-1.3	<i>Pseudomonas putida</i>	LC125152	++	42	42
20	Ue2-1.2	<i>Pseudomonas</i> sp.	–	++	42	42
21	Ue2-2.5.2	<i>Pseudomonas</i> sp.	LC125153	++	42	42
22	Oh-1.0.2	<i>Pseudomonas</i> sp.	LC125154	+	35	35
23	Oh-1.15	<i>Pseudomonas</i> sp.	LC125155	++	42	42
24	Fu2-2.1	<i>Vibrio</i> sp.	LC125156	++	42	42
Con Dao						
25	BD-2.3	<i>Achromobacter</i> sp.	LC125157	+	42	42
26	VB-2.1.2	<i>Brachybacterium rhamnosum</i>	LC125158	++	42	42
27	VB-1.8	<i>Gemella haemolysans</i>	LC125159	+	42	42
28	DT-2.15.1	<i>Gemella</i> sp.	–	++	42	42
29	VB-1.11	Gram + ve bacillus	–	+	39	42
–	DT-2.3.1	<i>Granulicatella adiacens</i>	–	++	42	42
30	AH-1.15.1	<i>Halomonas</i> sp.	LC125160	+	35	37
–	VB-1.5	<i>Klebsiella pneumoniae</i>	LC125161	+	42	42
–	VB-1.1	<i>Klebsiella pneumoniae</i>	LC125162	++	42	42
31	BD-2.4.2	<i>Microbacterium</i> sp.	LC125163	++	39	42
32	VB-1.14.2	<i>Microbacterium</i> sp.	LC125164	+	37	42
33	DT-2.3.2	<i>Microbacterium</i> sp.	LC125165	+	42	42
34	BD-2.5.3	<i>Ochrobactrum anthropi</i>	LC125166	++	42	42
35	BD-1.5.1	<i>Pseudomonas mendocina</i>	LC125167	++	42	42
36	AH-2.5.1	<i>Pseudomonas pseudoalcaligenes</i>	LC125168	+	42	42
37	BD-2.5.6	<i>Pseudomonas putida</i>	–	++	42	42
38	AH-2.1	<i>Pseudomonas stutzeri</i>	–	+	42	42
39	BD-2.2.1	<i>Pseudomonas stutzeri</i>	LC125170	+	42	42
40	BD-2.2	<i>Pseudomonas</i> sp.	LC125169	+	42	42
41	DT-1.15.2	<i>Yersinia</i> sp.	–	++	42	42

¹⁾ The numbers of the isolates that were used for biodegradation experiments. “–”, the isolate was discarded due to its pathogenic potential.²⁾ The oil degradation ability was surveyed by the color change of the incubation medium with INT : +, low ; ++, high.³⁾ The growth and survival of the isolates were examined after seven d of incubation at the temperatures of 15, 20, 30, 35, 37, 39, and 42°C.⁴⁾ The species were identified biochemically using the API kits.

percent) could degrade more than 50 percent of the oil present in the media. Among the Con Dao isolates, two isolates, No. 35 (BD-1.5.1, 66.3 percent) and No. 37 (BD-2.5.6, 62.9 percent) showed more than 50 percent degradation rates.

Biodegradation rates of *n*-alkanes by the isolates

The degradation rates of the *n*-alkanes, heptadecane and octadecane, by the isolates are shown in Fig. 4. All isolates could grow in the medium with the pure *n*-alkanes as the sole carbon sources. Among the 24 Iriomote isolates, 11 isolates showed degradation abilities of more than 50 percent for both chemicals. The highest degradation rates of heptadecane and octadecane were 92.0 and 93.1 percent for No. 23 (Ue2-2.5.2), followed by 88.5 and 78.4 percent for No. 6 (Oh-2.10.0) and 84.0 and 87.8 percent for No. 21 (Oh-1.15), respectively. Among the 17 Con Dao isolates, four isolates showed degradation abilities of more than 50

percent. The highest degradation rates of heptadecane and octadecane were 93.9 and 87.3 percent for No. 37 (BD-2.5.6), followed by 82.9 and 78.3 percent for No. 29 (VB-1.11) and 77.0 and 82.4 percent for No. 35 (BD-1.5.1), respectively.

Biodegradation of PAHs by the isolates

Fig. 5 shows the degradation rates of the PAHs, fluoranthene and pyrene, for the isolates. The degradation data for fluoranthene and pyrene showed different tendencies. Among the 24 isolates from Iriomote, 13 isolates could grow in the medium with fluoranthene. The highest degradation rate of 32.0 percent was shown by No. 11 (Fu2-3.2), followed by 29.8 percent shown by No. 8 (Ur-2.10.2) and 26.4 percent shown by No. 17 (Oh-1.15.2). Among the 17 Con Dao isolates, 13 isolates could grow in the medium with fluoranthene. The highest degradation rate of 28.4 percent was obtained with No. 34 (BD-2.5.3),

Fig. 3. Biodegradation rates of crude oil after 30 d of incubation with the isolates from Iriomote and Con Dao. The Iriomote isolates were numbered from 1 to 24 and those from Con Dao were numbered from 25 to 41. The numbers are the same as in Table 2. The bars represent standard deviations.

followed by 23.0 percent obtained with No. 33 (DT-2.3.2), and 22.6 percent obtained with No. 30 (AH-1.15.1).

Among the 24 Iriomote isolates, 15 isolates could grow in the medium with pyrene. The highest degradation ability of 39.0 percent was shown by No. 19 (Oh2-1.3), followed by 33.1 percent shown by No. 17 (Oh-1.15.2). Among the 17 Con Dao isolates, 12 isolates could grow in the medium with pyrene, and the highest degradation rate of 33.3 percent was shown by No. 31 (BD-2.4.2). Isolates No. 34 (BD-2.5.3) and No. 40 (BD-2.2) could degrade fluoranthene but not pyrene. On the other hand, No. 7 (Ue2-1.1) and No. 12 (Ur-2.5.1) could not degrade fluoranthene but degraded pyrene.

DISCUSSION

The percentages of ODB relative to the total bacterial counts obtained on Iriomote and Con Dao were 27.1 and 17.4 percent, respectively, in the S samples and 20.5 and 19.4 percent, respectively, in the W samples. The number of the isolates that degraded more than 50 percent of crude oil was higher on Iriomote (three isolates) than on Con Dao

(two isolates). It has been reported that oil-degrading bacteria accounted for 1–10 percent of the total number of bacteria in heterotrophic bacterial communities in vegetated soils (Atlas 1981). Because the sampling sites used in this study were exposed to no oil spill accidents during the collection period, the percentages of ODB (17.4–27.1 percent) indicate suitable application conditions for autochthonous bioaugmentation for the treatment of oil contamination in these areas. In addition, the relatively large number of ODB might also show chronic oil-contamination (Atlas 1981, Chikere et al. 2011) in the study islands from oil seepage of human activities or busy oil transportation.

The oil-degrading screening by INT based on the transfer of electron from NADH to the tetrazolium dye *p*-iodonitrotetrazolium violet. During the active growth of bacteria, an electron is transferred from NADH (colorless) to *p*-iodonitrotetrazolium violet resulting in a formazan dye (purple color). The darker medium color indicated better bacterial growth in the medium with oil. However, in some cases, high microbial growth were not indicative of higher degradation extents (Aldrett et al. 1997). Therefore, samples No. 3, 26, 28 and 31 did not show high biodegradability of

Fig. 4. Biodegradation rates of the *n*-alkanes after 30 d of incubation with the isolates from Iriomote and Con Dao.

The isolate numbers are the same as in Fig. 3.

The bars represent standard deviations.

Fig. 5. Biodegradation rates of the PAHs after 30 d of incubation with the isolates from Iriomote and Con Dao. The isolate numbers are the same as in Fig. 3. The isolates without columns mean no growth in the PAH medium. The bars represent standard deviations.

crude oil, though they indicated high activity in oil degradation screening (Table 2). The oil degradation rates shown by the isolates ranged from 26.2 to 71.2 percent for the Iriomote isolates and from 27.4 to 66.3 percent for the Con Dao isolates. In comparison with other studies, the oil degradation rates shown by our isolates were higher during the same incubation time than those obtained for other tropical areas such as Indonesia (4.9–22.0 percent; Chaillan et al. 2004) and India (11.6–52.8 percent; Mittal and Singh 2009).

The 45 ODB isolates, 25 from Iriomote and 20 from Con Dao, belonged to 18 genera. The isolate from the genus *Granulicatella* and the two unidentified isolates, a Gram-negative coccus and a Gram-positive bacillus, were isolated for the first time as ODB in this study. A *Granulicatella* isolate was identified by Al-Mailem et al. (2014) using a molecular method in man-made biofilms used for the bioremediation of hydrocarbons contaminating a sewage effluent. The prevalent genera of ODB are *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Burkholderia*, *Dietzia*,

Pseudomonas, and *Vibrio* (Atlas 1981, Chikere et al. 2011). The uncommon genera are *Brachy bacterium*, *Halomonas*, *Klebsiella*, *Microbacterium*, *Ochrobactrum*, *Paenibacillus*, and *Yersinia* (Daane et al. 2001, Hamme et al. 2003, Mnif et al. 2009, Rodrigues et al. 2009, Hassanshahian et al. 2012, Lily et al. 2013, Bhattacharya and Biswas 2014). The genus *Gemella* was first reported as ODB in 2015 (Kok Kee et al. 2015).

Among the 11 genera detected on Iriomote, the three dominant genera were *Achromobacter*, *Pseudomonas*, and *Ochrobactrum*. Among the 11 genera detected on Con Dao, the two dominant genera were *Pseudomonas* and *Microbacterium*. Three common genera, *Achromobacter*, *Ochrobactrum*, and *Pseudomonas*, were present in both study areas. *Pseudomonas* spp. are most frequently isolated and studied as hydrocarbon-degrading bacteria (Mittal and Singh 2009). Among the isolates from both study areas, those belonging to *Pseudomonas* species were not only dominant in quantity but also in their degradation efficiency. There were four members of *Pseudomonas* species that

gave more than 50 percent of oil degradation. Because *Pseudomonas* species play a significant role in oil degradation due to their wide distribution and ability to degrade a wide range of hydrocarbon compounds, they are studied for genetic diversity in degradation functions and gene modification and used as biosensors and bioremediation agents (Palleroni et al. 2010).

n-Alkanes are major components in oil and readily degraded in the environment via several pathways (Hassanshahian et al. 2012). Among crude oil components, *n*-alkanes are the preferred carbon source for bacteria (Delille et al. 2004). This is the reason why the degradability of the *n*-alkanes by the isolates (Fig. 4) was more distinct than the one of crude oil (Fig. 3). The degradation rate of an *n*-alkane depends on the chain length. Short-chain alkanes ($<C_9$) are volatilized and rapidly released into the atmosphere. Intermediate-chain alkanes (C_{10} – C_{24}) are readily degraded by microorganisms, and longer-chain alkanes are generally resistant to degradation. The results of this study showed that the isolates with a high degrading ability for oil also showed a high degrading ability for heptadecane (C_{17}) and octadecane (C_{18}). The number of the isolates that degraded more than 50 percent of the *n*-alkanes present in the media was greater on Iriomote (11 isolates) than on Con Dao (four isolates). The members of the genera *Pseudomonas* and *Acinetobacter* were good *n*-alkane degraders, which has also been reported previously (Chikere et al. 2011).

PAHs belong to most hazardous organic pollutants, which is due to their toxicogenic, mutagenic, and carcinogenic properties (Darmawan et al. 2015). PAHs are present in oil, are highly resistant to biodegradation, and are degraded by fewer microbial species as compared with oil or alkane compounds (Chaerun et al. 2004). In this study, only 13 and 15 isolates out of the 24 isolates from Iriomote and 13 and 12 isolates out of the 17 isolates from Con Dao could grow in the medium with fluoranthene and pyrene, respectively. The number of the isolates that degraded more than 25 percent of the fluoranthene and pyrene present in the media was greater on Iriomote (two isolates each) than on Con Dao (one isolate each). The isolates that showed high abilities to degrade the PAHs were different from those with high abilities to degrade oil or the alkanes. Because of the variety and complexity in the structure of PAHs, there are diverse catabolic pathways according to microbial species. The major principle of aromatic hydrocarbon biodegradation include two steps: 1) activating a thermodynamically stable benzene ring from structurally diverse aromatics by adding oxygen atoms of molecular oxygen as hydroxyl groups to the aromatic ring; and 2)

subsequent cleavage, ring-cleavage dioxygenases decyclize aromatic compounds allowing the products to be channeled into the cell's central metabolic pathways (Pérez-Pantoja et al. 2010, Haddock 2010). Key enzymes in aerobic aromatic degradation are oxygenases. In cases of fluoranthene and pyrene, there are 54 and 27 enzymes, respectively, present in *Mycobacterium vanbaalenii* involve in the degradation of those PAHs (Kweon et al. 2010).

Isolates No. 6 (Oh-2.10.0) and No. 35 (BD-1.5.1) showed high abilities to degrade oil and the alkanes, but they could not grow in the medium with the PAHs. Isolates No. 17 (Oh-1.15.2) from Iriomote and No. 30 (AH-1.15.1) and No. 33 (DT-2.3.2) from Con Dao showed high degradation rates for fluoranthene but low degradation rates for oil and the alkanes. Isolates No. 17 (Oh-1.15.2) from Iriomote and No. 31 (BD-2.4.2) from Con Dao showed high abilities to degrade pyrene but low abilities to degrade oil and the alkanes. A number of hydrocarbon-degrading enzymes from bacteria participate in the degradation of different groups of hydrocarbon compounds in oil. Each bacterial group has only one or a few enzymes that can degrade one or a group of hydrocarbon compounds. It is noteworthy that isolates No. 19 (Oh2-1.3) and No. 34 (BD-2.5.3) were able to degrade more than 40 percent of the oil and *n*-alkanes, and more than 25 percent of the PAHs.

Here, the temperature resistance of the isolates was studied. At higher temperatures, the degradation rate generally increases because of the increase in activity of microorganisms (Das and Chandran 2011), and the optimum temperatures range from 30 to 40°C in soil environments (Atlas 1981). In this study, all isolates could grow at 35°C, among which 21 isolates out of the 25 isolates from Iriomote and 16 isolates out of the 20 isolates from Con Dao could even grow at 42°C. These temperature-resistant isolates might show potential for bioremediation applications, especially in summer time (Hemalatha and Veeramanikandan 2011). The temperature resistance, however, may lead to potential pathogenicity to humans and other warm-blooded animals. Isolates that are characterized as good oil-degraders but are also pathogens have to be excluded from field use. Some bacteria that are close relatives of the isolates from this study have been listed as pathogens, including *B. cepacia*, *G. adiacens*, and *K. pneumoniae* (Govan et al. 1996, Podschun and Ullmann 1998, Cargill et al. 2012).

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