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Mercury (Hg)-Resistant Bacteria in Hg-Polluted Gold Mine Sites of Bandung, West Java Province, Indonesia

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In the present study, ten mercury-resistant heterotrophic bacterial strains were isolated from mercury-contaminated gold mine sites in Bandung, West Java Province, Indonesia. The bacteria (designated strains SKCSH1- SKCSH10) were capable of growing well at ~200 ppm of $HgCl_2$ except for strain SKCSH8, which was able to grow at 550 ppm $HgCl_2$. The bacteria were mesophilic and grew optimally at 1% NaCl at neutral pH with the optimal growth temperature of 25-37 °C. Phenotypic characterization and phylogenetic analysis based on the 16S rRNA gene sequence indicated that the isolates were closely related to the family *Xanthomonadaceae*, *Aeromonadaceae*, and *Pseudomonadaceae* and they were identified as *Pseudomonas* spp., *Stenotrophomonas* sp., and *Aeromonas* sp. Eight bacterial strains were shown to belong to the *Pseudomonas* branch, one strain to the *Stenotrophomonas* branch and one strain to the *Aeromonas* branch of the γ-Proteobacteria. Phylogeny based on their 16S rRNA gene sequences indicated that four of the isolates (SKCSH1, SKCSH4, SKCSH7, SKCSH9) could be classified as representatives of four novel species in the genus *Pseudomonas* that were allocated to *P. moraviensis* (96.96% similarity) and *P. plecogossicida* (94.53, 96.61, and 96.73% similarity). Four other isolates could be allocated to *P. plecogossicida* (97.57 and 98.66% similarity) and *P. hibiscicola* (99.97% similarity), one isolate to *Stenotrophomonas africana* (99.69% similarity), and one other isolate to *Aeromonas hydrophila* subsp. *ranae* (99.43% similarity). The findings of this study provide the first information of the phylogenetically-diverse Hg-resistant bacteria in the Hg-polluted sites of Indonesia that may be highly useful for developing *in situ* bioremediation or detoxification of Hg-contaminated sites in Indonesia.

Key words: 16S rRNA gene sequences, *in situ* bioremediation, Mercury (Hg), phenotype, phylogenetic analysis

Tujuan penelitian ini adalah untuk mempelajari secara filogenetika bakteri-bakteri heterotrof yang resisten terhadap merkuri (Hg) yang diisolasi dari daerah pertambangan emas yang tercemar Hg di Bandung, Jawa Barat, Indonesia. Ada 10 isolat bakteri resisten Hg yang telah diisolasi dari daerah pertambangan tersebut. Kesepuluh strain adalah mesofilik dan mampu tumbuh pada LB medium dengan konsentrasi $HgCl_2$ sampai sekitar 200 mg L^{-1} (kecuali strain SKCSH8 mampu tumbuh sampai konsentrasi $HgCl_2$ sebesar 550 mg L^{-1}) dengan kondisi pertumbuhan optimum: konsentrasi NaCl 1%, pH netral, dan pada kisaran suhu 25-37 °C. Karakterisasi secara fenotip dan analisis sekuen gen 16S rRNA menunjukkan bahwa delapan strain bakteri termasuk dalam genus *Pseudomonas*, satu strain dalam genus *Stenotrophomonas*, dan satu strain lagi dalam genus *Aeromonas* dari kelas γ-Proteobacteria. Analisis filogenetika dari sekuen gen 16S rRNA mengindikasikan bahwa ke-empat isolat (SKCSH1, SKCSH4, SKCSH7, SKCSH9) mempunyai peluang sebagai spesies baru dari genus *Pseudomonas* yaitu *Pseudomonas moraviensis* (dengan similaritas 96,96%) dan *Pseudomonas plecogossicida* (dengan kemiripan 94%, 53%, 96,6%, dan 96,73%). Empat isolat yang lain mempunyai kesamaan gen 16S rRNA sebesar 97,57% dan 98,66% terhadap *Pseudomonas plecogossicida* dan sebesar 99,97% terhadap *Pseudomonas hibiscicola*, serta dua isolat lagi mempunyai kesamaan gen 16S rRNA sebesar 99,69% terhadap *Stenotrophomonas africana* dan sebesar 99,43% terhadap *Aeromonas hydrophila* subsp. *ranae*. Hasil penelitian ini memberikan informasi pertama tentang keanekaragaman secara filogenetika bakteri resisten Hg pada lahan yang tercemar Hg di Indonesia, yang mungkin dapat bermanfaat dalam mengembangkan bioremediasi secara in situ lahan-lahan yang tercemar Hg di Indonesia.

Kata kunci: analisis filogenetika, bioremediasi *in situ*, fenotip, merkuri (Hg), sekuen gen 16S rRNA

Mercury (Hg) pollution is a significant problem in traditional mining, in particular that located in Indonesia, with respect to abandoned Hg mines and residual Hg from gold mining operations that largely

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contribute a significant loading of Hg to the watersheds each year, e.g. traditional gold mining in Bunikasih, Pangalengan, West Java Province, Indonesia. Correspondingly, mercury is known as one of the most toxic heavy metals and its toxicity is due to its ability to bind readily to thiol group of protein, thus inactivating vital cell functions (Wagner-Dobler 2003). Most of the Hg in the atmosphere is in the form of elemental Hg (Hg^0), which is volatile and is oxidized to the mercuric ion (Hg^{2+}) as a result of its interaction with ozone in the presence of water (Chiu *et al*. 2007; Zhu *et al*. 2008). The main form of Hg in the aquatic environment is Hg^{2+} (Green-Ruiz 2006; Ariya *et al*. 2009; Mason 2009). When both inorganic mercury forms (Hg^{2+} and Hg^0) are present in aquatic systems, they are converted into highly toxic organic mercury (methylmercury (MeHg)) that is subsequently bioaccumulated through all levels of the food chain (Mason 2009). Thus, the bioaccumulation and biomagnification of mercury in food chains pose a risk to consumers at the upper trophic levels (Garcia-Sanchez *et al*. 2006; Ni Chadhain *et al*. 2006; Yang *et al*. 2009).

Importantly, mercury contamination caused by the amalgamation of gold in small-scale gold mine regions is also a current environmental problem worldwide, particularly in the mining areas of developing countries. The mining operations generally involve the extensive use of mercury to recover gold through the amalgamation process (Ambers and Hygelund 2001; Lecce *et al*. 2007; Tian *et al*. 2009). The open-pan amalgamation, when pasty amalgam is heated to vaporize the mercury and separate the gold which is left behind, causes leaving residual mercury to be left in the gold mining sites. Up to the present, mercury contamination of stream sediments caused by gold mining operations have been reported on a worldwide basis (Ambers and Hygelund 2001; Miller *et al*. 2003; Lecce *et al*. 2007; Tian *et al*. 2009). Although mercury has been known as an environmental pollutant for many decades, the release of mercury to the environment from anthropogenic sources is of growing concern (US EPA, 2003). In North Sulawesi Province of Indonesia, in 1998 approximately 22000 small-scale gold miners were active and produced an estimated 10 tonnes of gold bullion. The mercury concentration which was employed in this operation reached 1 kg out of 30 kg of ore (Ayhuan *et al*. 2003).

From a microbiological standpoint, mercury-resistant bacteria, which include aerobes or facultative anaerobes, are frequently isolated from a variety of environmental niches such as water, soil and sediment (Barkay *et al*. 2003; Chiu *et al*. 2007). They have an essential role in mercury bio-geochemistry and recycling. The mechanism of their mercury resistance is mediated through a cytoplasmic mercuric reductase by which soluble Hg^{2+} is converted into insoluble Hg^0, followed by volatilization of the relatively non-toxic Hg^0. The mercuric reductase is encoded by the *merA* gene (Nascimento and Chartone-Souza 2003; Fantozzi *et al*. 2009). Essa *et al*. (2002) reported that several different mechanisms can make microbes able to survive in the presence of high concentrations of Hg. By having evolved resistance mechanisms to detoxify several chemical forms of mercury, resistant microbes may play an important role in mercury bio-geochemistry in mercury-contaminated environments (Dzairi *et al*. 2004; Gustin and Stamenkovic 2005; Ni Chadhain *et al*. 2006). Thus, it is expected that exploration of mercury-resistant microbes may potentially be beneficial for detoxifying mercury-contaminated sites.

Hence, the aim of this work was to study the phylogenetic diversity of indigenous Hg-resistant bacterial strains at a traditional gold mine of Indonesia combined with phenotypic analysis (the so-called polyphasic characterization). It is expected that the molecular ecological approaches to studying the 16S rRNA gene would exhibit the presence of phylogenetically novel bacterial sequences at these sites as well as novel bacterial isolates resistant to Hg. Such information may be highly useful for developing in situ bioremediation or detoxification of Hg-contaminated sites in Indonesia.

MATERIALS AND METHODS

Study Sites, Sample Collection, and Physicochemical Analysis. The study site was located at a traditional gold mining in highly Hg-contaminated environment and overburdened site in Bunikasih, Pangalengan, West Java Province, Indonesia. River sediment samples were collected from 20 random positions of the Bunikasih River, Pangalengan (coded as sediment I-IV) and one soil sample from tailings (coded as tailing soil) in November 2009. For river sediment samples, the site was divided into four plots. At each plot one composite mixed sample was obtained by pooling 4-6 sediment samples from random locations throughout the plot. All samples were transferred to the laboratory and stored at -20 °C until required for analysis. Sample pH was measured by potentiometer in suspension (1:1 w/v) according to

DIN ISO 10390 (DIN ISO 1997), and total mercury content was analyzed according to DIN ISO 38414-S (DIN ISO 1983).

Isolation and Cultivation of Mercury (Hg)-Resistant Bacteria. Bacterial strains were isolated from both samples (sediment and tailing soil) on Luria Bertani (LB) broth supplemented with three concentrations of Hg^{2+} as $HgCl_2$ (25, 50, 100 mg L^{-1}) at pH 7 and supplemented with antifungal (30 mg L^{-1} nystatin) to prevent fungal growth. The flasks were incubated at room temperature (25 °C) for 4 d with agitation of 120 rpm, and samples were subsequently transferred onto LB agar plates supplemented with 25 mg L^{-1} of $HgCl_2$ using a 4-way streak method. Ten morphologically different colonies were randomly chosen from each type of Hg-amended agar plates and subcultured on Hg-supplemented LB agar six times for 3-4 d to purify the isolates. Eventually, the pure cultures were subcultured onto Hg-amended LB agar plate and Hg-amended LB broth for stock cultures.

Minimum Inhibitory Concentration (MIC) of Hg^{2+}. The assay aimed for bacterial growth at the highest Hg^{2+} concentration in the form of $HgCl_2$. Briefly, bacterial cultures in LB broth were transferred to a fresh medium and supplemented with $HgCl_2$ at concentrations ranging from 25 to 600 mg L^{-1}. The cultures were then incubated at room temperature and their optical density (OD) was measured at the time of inoculation and afterward up to 4 d with spectrophotometer at λ at 600 nm. Minimal inhibitory concentration (MIC) was the minimum Hg^{2+} concentration that inhibited growth, i.e., no increase in λ at 600 nm in 4 d.

Phenotypic Characterization. Phenotypic characterization was conducted on the analysis of bacterial cell morphology, biochemical tests, tests of the ability to grow on the variations of temperature, salinity, and pH (Cappuccino and Sherman 2005). All strains were tested at 25 °C for the following key characteristics. Analysis of bacterial cell morphology used Gram stain, endospore staining and capsule staining, where bacteria were viewed routinely by phase contrast microscopy at ×1000 magnification. Biochemical tests included utilization of catalase, oxidase and citrate, indole production, H_2S production, MRVP test (Methyl Red-Voges Proskaur), urease test, nitrate reduction and hydrolysis of gelatin, casein, lipid, starch and tween 80. In addition, tests of the bacterial ability to use different sugars as a carbon source were performed. These included glucose, galactose, fructose, mannosa, arabinose, sucrose, maltose, and lactose. All strains were tested for their ability to grow on LB agar at various temperatures (4, 25, 37, and 50 °C) and on LB broth at different pH values (3, 5, 8, and 10) adjusted by adding 1N HCl or 1N NaOH. Tolerance to salinity was determined in Nutrient Agar (NA) supplemented with 0.5, 3, and 5% NaCl.

Phylogenetic Characterization of Hg-Resistant Bacterial Strains. Chromosomal DNA for phylogenetic characterization was extracted and purified by using the UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.) according to the manufacturer's instructions. The 16S rRNA gene was amplified by PCR with primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') as described previously (Chaerun *et al*. 2004). The 16S rRNA gene was amplified from genomic DNA by PCR (2720 Thermal Cycler Applied Biosystems) in a final volume of 25 µL, consisting of 2.5 µL of 10 x Kapa Taq buffer, 5 µL of 1 mM deoxynucleoside triphosphate (dNTPs), 1 µL of 10 µM each of primers 27F and 1492R, 0.1 µL of Kapa Taq DNA polymerase, 1 µL of the template DNA, and 14.4 µL of deionized H_2O. Amplifications were carried out at 95 °C for 3 min, 30 cycles at 95 °C for 1 min, 56 °C for 45 s, 72 °C for 1 min 30 s, and the final extension for 4 min at 72 °C. PCR products were checked by 1% agarose gel electrophoresis and stained with ethidium bromide (EtBr). The amplified products were purified and sequenced by the direct sequencing method at the 1st Base Bioengineering Technology Service Co., Ltd. (Singapore). The sequences of the 16S rRNA genes from each isolate were used as query to determine the genus and species of its closest prokaryotic relative using BLASTN (Altschul *et al*. 1990). Sequences were aligned using CLUSTAL X program (Version 1.83) (Thompson *et al.* 1994). Phylogenetic trees were inferred by the neighbour-joining method (Saitou and Nei 1987) with the phylogenetic analysis package PAUP* 4.0 (Swofford 2002) and TREEVIEW was utilized to plot the tree topologies. A bootstrap analysis was performed using 1000 trial replications to provide confidence estimates for branch support (Felsenstein 1985). Reference 16S rRNA sequences were obtained from the GenBank that were included in the phylogenetic analysis.

RESULTS

Sample Characteristics (pH and Hg Concentration). Sample characteristics were performed to provide a detailed description of sampling

Table 1 pH and mercury (Hg) concentration of samples

Characteristics	Sampling site				
	Sediment I	Sediment II	Sediment III	Sediment IV	Tailing soil
pH	7.8	7.9	7.8	7.4	8.2
Hg conc. (mg L^{-1})	28.4	46.1	44	60.7	27.6

Table 2 Mercury-resistant bacterial strains and their minimum inhibitory concentrations (MIC) of Hg^{2+} from the Bunikasih traditional gold mine, Pangalengan, West Java Province, Indonesia

Isolates	MIC of $HgCl_2$ (mg L^{-1})	Sampling Site
SKCSH1	250	Tailing soil
SKCSH2	225	Sediment II
SKCSH3	175	Sediment III
SKCSH4	225	Sediment IV
SKCSH5	100	Sediment IV
SKCSH6	60	Sediment IV
SKCSH7	80	Sediment IV
SKCSH8	550	Sediment II
SKCSH9	250	Sediment IV
SKCSH10	200	Sediment IV

site conditions with respect to the pH and Hg concentration that are listed in Table 1. Both samples (sediments and tailing soil) had slightly alkaline pHs (7.4-8.2). Both samples were also characterized by high Hg concentrations (27.6-60.7 mg L^{-1}), suggesting that the bacteria residing the site might be resistant to Hg. The highest Hg concentration was found in Sediment IV and the lowest was obtained in the tailing sample (Table 1).

Isolation and Cultivation of Hg-Resistant Bacteria and Their Minimum Inhibitory Concentrations (MIC) of Hg^{2+}. Of the sediment and tailing soil samples, ten morphologically different bacterial colonies were successfully isolated and they were designated as strains SKCSH1-SKSH10 (Table 2). Only one isolate was obtained from tailing soil sample and from sediment III. The more highly polluted samples (sediment II and IV) had the highest number of mercury-resistant aerobic heterotrophs (Table 2). Two isolates (strains SKCSH2 and SKCSH8) were recovered from sediment II and six isolates (strains SKCSH4-7 and SKCSH9-10) from sediment IV whose sediment mercury concentrations were 46.1 and 60.7 mg L^{-1}, respectively. In the case of the tailing soil, only one isolate was identified due to the appearance of the same colonies on agar plates. Moreover, no Hg-resistant bacterial strains were able to be isolated from sediment I. The failure to isolate bacterial strains in this sample was because the colonies growing on agar plates were dominated by fungal colonies (data not shown). A high level of resistance to $HgCl_2$ was noted with all bacterial strains, capable of growing in the presence of at least 25 mg L^{-1} of $HgCl_2$ with the most resistant strain growing in the presence of 550 mg L^{-1} of $HgCl_2$ (Table 2). Of the ten isolates, six isolates were able to grow at ≥ 200 mg L^{-1} of $HgCl_2$ (one strain was highly resistant to $HgCl_2$ of 550 mg L^{-1}, while the four remaining isolates grew below 200 ppm of $HgCl_2$). Strain SKCSH8 (from sediment II) was the most resistant strain to $HgCl_2$. The most sensitive strains were found for strains SKCSH6 and SKCSH7 (from sediment IV), which were inhibited by 60 and 80 mg L^{-1} of $HgCl_2$, respectively (Table 2).

Phenotypic Characterization of Hg-Resistant Bacteria. Phenotypic characteristics of all ten Hg-resistant bacterial strains are given in Table 3. All ten strains (SKCSH1~SKCSH10) were Gram-negative and had no capsule. Cells of all strains were rod-shaped in all growth phases in both liquid and agar media, except for strains SKCSH7 and SKCSH8 which were coccoid and curved-rods, respectively. When they were grown on LB agar, colonies of all strains were white, except that strain SKCSH7 which was yellow-colored. Catalase activity was present for all strains, while oxidases were only present for strains SKCSH1, SKCSH4, SKCSH7, SKCS8, and SKCSH9. Negative results were obtained for indole production, H_2S production from thiosulfate, and urease activity, and positive results for VP reaction for all strains. Assays for MR reduction were negative for all strains with the exception of strains SKCSH1 and SKCSH4. Citrate utilization was also negative for all strains except for strains SKCSH1, SKCSH2, SKCSH4, and SKCSH9. Hydrolysis of lipid and tween 80 was detected for all bacterial strains, while none of them could hydrolyze gelatin with the exception of strain SKCSH2. Of the ten strains, only strains SKCSH2 and SKCSH7 were able to hydrolyze casein. Likewise, only four strains (SKCSH2, SKCSH4, SKCSH8, and SKCSH9) were capable of reducing nitrate to nitrite. NaCl tolerance was also studied with NaCl added at final

Table 3 Phenotypic characteristics of mercury-resistant bacterial strains isolated from the Bunikasih traditional gold mine, Pangalengan, West Java Province, Indonesia

Characteristics	Isolates									
	1	2	3	4	5	6	7	8	9	10
Gram	-	-	-	-	-	-	-	-	-	-
Capsules	-	-	-	-	-	-	-	-	-	-
Cell shape	rods	rods	rods	rods	rods	rods	cocci	curved rods	rods	rods
Colony colour	white	white	white	white	white	white	yellow	white	white	white
Catalase Activity	+	+	+	+	+	+	+	+	+	+
Oxidase Activity	+	-	-	+	-	-	+	+	+	-
IMViC Test:										
Indole Production	-	-	-	-	-	-	-	-	-	-
MR Reduction	+	-	-	+	-	-	-	-	-	-
VP Reaction	+	+	+	+	+	+	+	+	+	+
Citrate Utilization	+	+	-	+	-	-	-	-	+	-
H_2S Production	-	-	-	-	-	-	-	-	-	-
Urease Activity	-	-	-	-	-	-	-	-	-	-
Hydrolysis of :										
Gelatin	-	+	-	-	-	-	-	-	-	-
Starch	-	-	-	-	-	-	-	-	-	-
Casein	-	+	-	-	-	-	+	-	-	-
Lipid	+	+	+	+	+	+	+	+	+	+
Tween 80	+	+	+	+	+	+	+	+	+	+
Nitrate reduction to nitrite	-	+	-	+	-	-	-	+	+	-
Growth at/in :										
0.5% NaCl	+	+	+	+	+	+	+	+	+	+
3% NaCl	+	+	+	+	+	+	+	+	-	+
5% NaCl	+	+	-	+	-	-	+	-	-	-
4°C	-	-	-	-	-	-	-	-	-	-
25°C	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+
55°C	-	-	-	w	-	w	w	w	w	-
Growth on pH:										
3	-	-	+	-	-	-	-	-	-	-
5	-	+	++	+++	-	++	-	-	+	+++
8	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
10	+	+	+	+	+	+	+++	-	+++	-
Carbon source:										
Glucose	+(A)	-	-	-	-	-	-	-	-	-
Galactose	-	-	-	+(A)	-	+(A)	+(A)	-	-	-
Fructose	-	-	-	-	-	-	-	-	-	-
Mannose	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-

Note: (-): negative result; (+): positive result; (++): high growth; (+++): extremely high growth; (w): weak growth; (A) Acid production; (MR): methyl red; (VP): Voges-Proskauer; (Isolates 1-10): SKCSH1-SKCSH10

concentrations in the range of 0.5-5% (w/v). All strains were able to grow at NaCl levels of 0.5-3% NaCl (optimum 1% NaCl), except for strain SKCSH9 which did not grow at 3% NaCl. Moreover, no growth occured at concentrations greater than 3%, with the exception of strains SKCSH1, SKCSH2, SKCSH4, and SKCSH7.

Table 4 Mercury-resistant bacterial strains isolated from the Bunikasih traditional gold mine, Pangalengan, West Java Province, Indonesia

Sampling sites	Strain	Species	Sequence similarity (%)
Tailing Soil	SKCSH1	*Pseudomonas moraviensis*	96.96
Sediment II	SKCSH2	*Stenotrophomonas africana*	99.69
Sediment III	SKCSH3	*Pseudomonas plecogossicida*	97.57
Sediment IV	SKCSH4	*Pseudomonas plecogossicida*	94.53
Sediment IV	SKCSH5	*Pseudomonas plecogossicida*	98.66
Sediment IV	SKCSH6	*Pseudomonas hibiscicola*	99.97
Sediment IV	SKCSH7	*Pseudomonas plecogossicida*	96.61
Sediment II	SKCSH8	*Aeromonas hydrophila* subsp. *ranae*	99.43
Sediment IV	SKCSH9	*Pseudomonas plecogossicida*	96.73
Sediment IV	SKCSH10	*Pseudomonas hibiscicola*	99.97

The name of bacterial strains was a result of 16S rRNA sequencing analysis.

All strains grew at 25 and 37 °C, with no or weak growth at 55 °C. Most strains grew over a pH range of 5 -10 (optimum pH 7-8), but none of strains did grew at pH 3 (below pH 5) with the exception of strain SKCSH3. In addition, all strains were incapable of producing acid from the oxidation or the fermentation of the following carbohydrates: gluclose, galactose, fructose, mannose, arabinose, sucrose, maltose and lactose. However, some strains had the ability to produce acid from oxidation or fermentation of glucose (SKCSH1) and galactose (SKCSH4, SKCSH6, SKCSH7).

Phylogenetic Analysis of Hg-Resistant Bacteria. In order to identify the bacterial isolates, their 16S rRNA genes were amplified and sequenced. Strain SKCSH1 was affiliated to *Pseudomonas moraviensis* (96.96% similarity), strain SKCSH2 to *Stenotrophomonas africana* (99.69% similarity), strains SKCSH3, SKCSH4, SKCSH5, SKCSH7, SKCSH9 to *Pseudomonas plecogossicida* (97.57%, 94.53%, 98.66%, 96.61%, 96.73% similarity, respectively), strains SKCSH6 and SKCSH10 to *Pseudomonas hibiscicola* (99.97% similarity), and strain SKCSH8 to *Aeromonas hydrophila* subsp. *ranae* (99.43% similarity) (Table 4). In an inferred phylogenetic tree, these three novel species (SKCSH4, SKCSH7, SKCSH9) together with our two other bacterial strains (i.e., SKCSH3 and SKCSH5) formed one distinct phylogenetic which was distantly related to recognizable species of the genus *Pseudomonas*, with *Pseudomonas plecogossicida* as the closest relative (Fig 1). The 16S rRNA gene sequences of each of the five strains were more similar to each other (> 99%) than to any other sequence in GenBank. In addition, strain SKCSH1 branched off most distantly and placed this strain within the genus *Pseudomonas* with *Pseudomonas moraviensis* as its closest neighbour. The 16S rRNA gene sequences of strains SKCSH6 and SKCSH10 all had the signature nucleotides and nucleotide pairs to indicate that they belonged to the family *Pseudomonadaceae*. The 16S rRNA gene sequences from strains SKCSH6 and SKCSH10 were almost 100% identical to each other, and were 99.97% identical to that of *Pseudomonas hibiscicola*. Phylogenetic analyses placed these two bacteria in a group that included our other Hg-resistant bacterial strain (SKCSH2), *Pseudomonas hibiscicola*, *Pseudomonas geniculata*, *Stenotrophomonas maltophilia* and *Stenotrophomonas africana* (Fig 2). Strain SKCSH2 belonged to the family *Xanthomonadaceae* and the 16S rRNA gene sequence of this strain was 99.69% identical to that of *Stenotrophomonas africana*. These three bacteria (strains SKCSH2, SKCSH6 and SKCSH10) grouped together with more than 99% similarity. In addition, strain SKCSH8 formed a distinct phylogenetic group of the family *Aeromodaceae* within the genus *Aeromonas* with *Aeromonas hydrophila* subsp. *ranae* as its closest neighbour (99.43% sequence similarity) (Fig 3).

DISCUSSION

All Hg levels in samples taken were above the maximum permissible concentration of Hg in sediments and soils of 0.04 mg L^{-1} (Swedish Environmental Protection Agency 1967), while the maximum permitted Hg concentration in water is 0.005 mg L^{-1} (Government Decree of the Republic of Indonesia No. 82, 2001). Hence, methods for cleaning up mercury-polluted environments in a cost-effective way are urgently required, since mercury is a highly toxic metal. Thus, by finding the mercury-resistant bacteria in this study, they might be beneficial to be

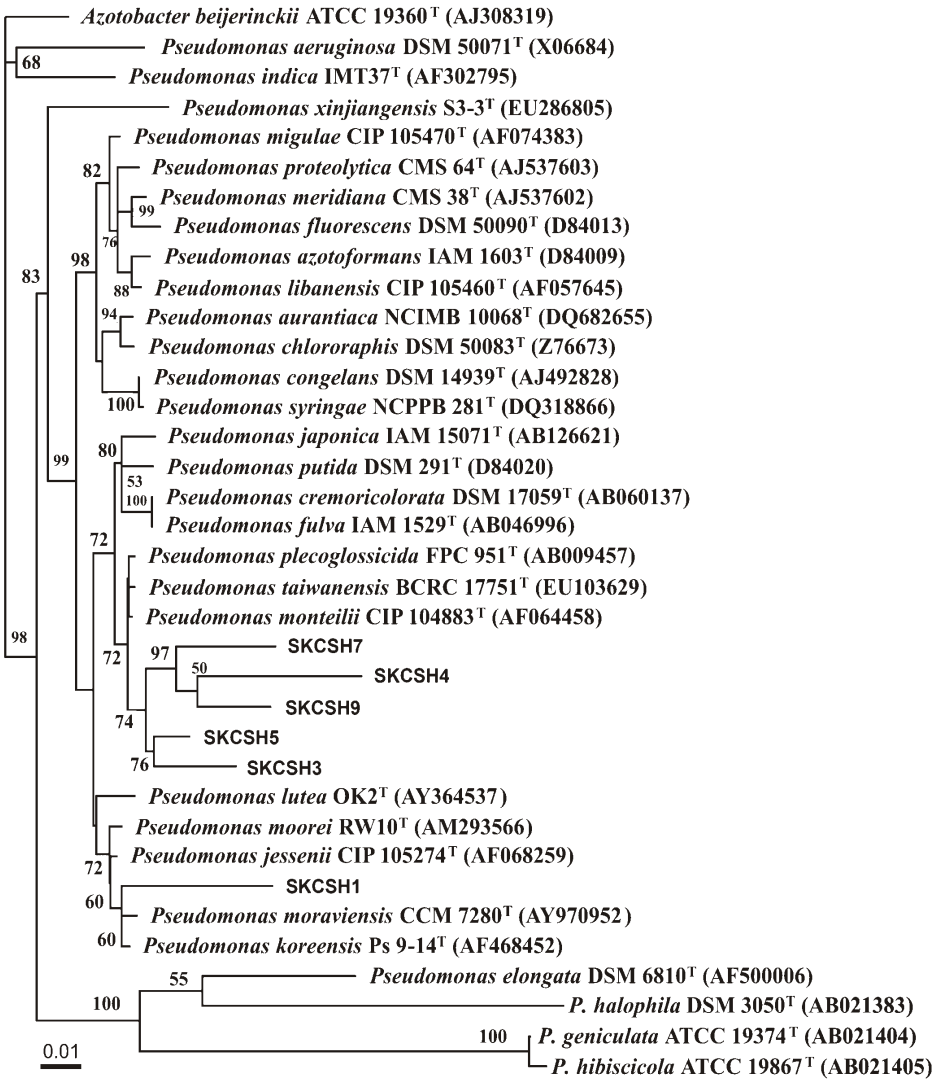

Fig 1 Phylogenetic tree of strains SKCSH1, SKCSH3, SKCSH4, SKCSH5, SKCSH7, and SKCSH9, based on 16S rRNA gene sequences. The branching pattern was produced by the neighbour-joining method. Numbers at nodes are percentage bootstrap values based on 1000 iterations; only values above 50% are shown. GenBank accession numbers are given in parentheses. Bar, 1 substitutions per 100 nucleotides.

biological agents for environmental applications. Application of these bacteria to mercury-polluted environments may lead to a potential cleanup technology which may be capable of bioremediating soil, water, or sediments contaminated with mercury in an environmentally friendly way. The role of the bacteria in mercury bioremediation is likely to have high levels of efficacy due to their mer operon-based resistance mechanism, which functions by active enzymatic reduction of mercury ions to water-insoluble metallic mercury (Vetriani *et al*. 2005; Omichinski 2007; Poulain *et al*. 2007). Therefore, further investigation of these bacterial mercury resistance is greatly needed in an effort to develop environmentally friendly, cost-effective bioremediation technology in Indonesia. Correspondingly, current environmental issues regarding mercury contamination in Indonesia are due to the illegal mining of gold using the amalgam process in which mercury is employed. Both wastewater and waste produced in the amalgam process were discharged into rivers, where mercury can be persistent over long periods of time, which in turn is a risk to humans because of its accumulation in the food chain (Laperdina 2002; Gustin *et al*. 2003; Wang *et al*. 2004; Kelly *et al*. 2006). The persistence of mercury in these mining disposals would enable us to acquire a high diversity of mercury-resistant bacteria. From the sediment and tailing soil samples of the Bunikasih River from which our mercury-resistant bacteria were isolated, ten heterotrophic bacteria were isolated with their level of resistance to mercury ($HgCl_2$) of 60-550 mg L^{-1} (Table 2). Their mercury resistance, which was over the Hg concentrations of the samples (27.6-60.7 mg L^{-1}) (Table 1), indicates that the bacteria might have adapted to mercury accumulated in sediments of the Bunikasih River for a long period of time due to their

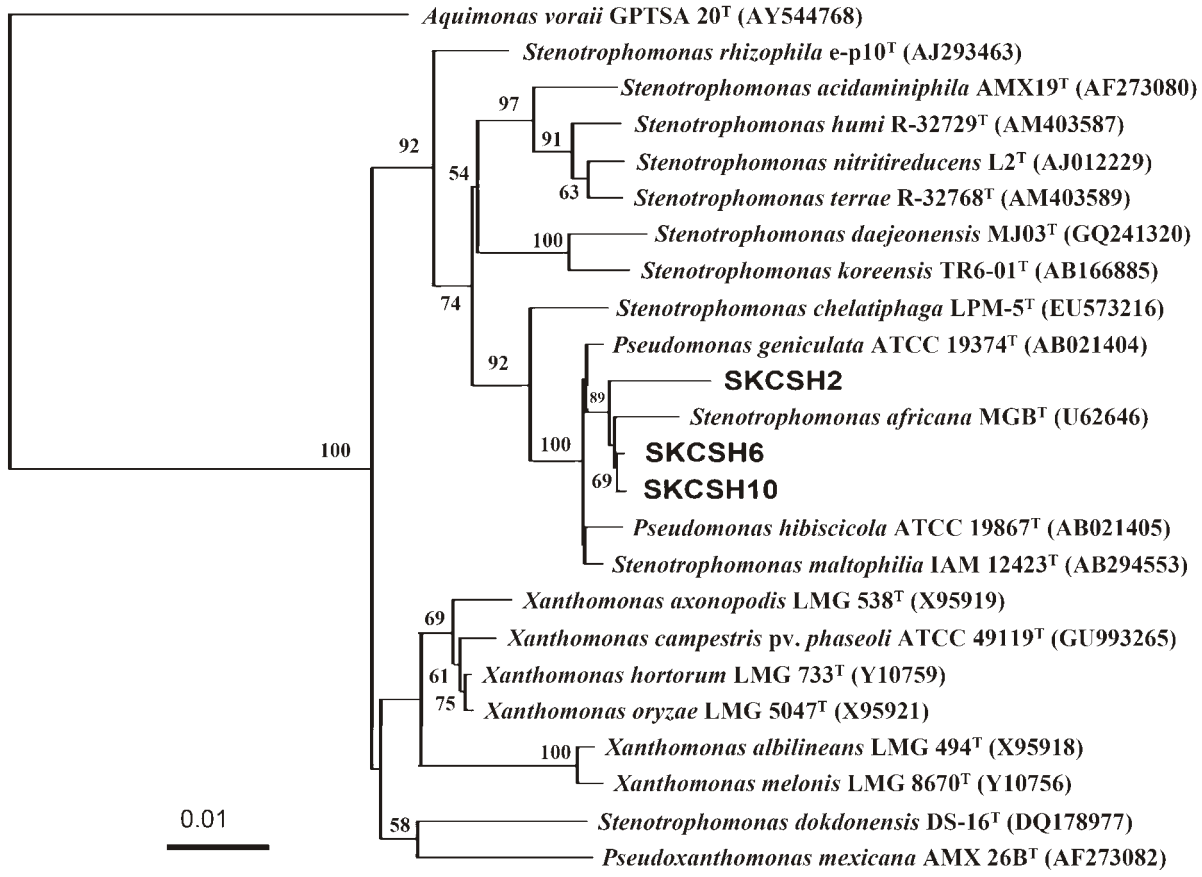

Fig 2 Phylogenetic tree of strains SKCSH2, SKCSH6, and SKCSH10, based on 16S rRNA gene sequences. The branching pattern was produced by the neighbour-joining method. Numbers at nodes are percentage bootstrap values based on 1000 iterations; only values above 50% are shown. GenBank accession numbers are given in parentheses. Bar, 1 substitutions per 100 nucleotides.

superior traits (i.e. a high and constitutive expression of the mercury resistance genes) (Omichinski 2007). In MIC experiments (Table 2), the bacterial strains were grown on LB medium containing ionic mercury ($HgCl_2$), where their growth greatly depended on the Hg(II) bioavailability and toxicity. Nutrients in LB medium that contained sulfhydryl group (i.e. yeast extract) and a negatively charged ion (i.e. chloride) which binds to ionic mercury, thus altering its bioavailability and toxicity (Essa *et al*. 2002; Barkay *et al*. 2003). Therefore, the ability to grow in the presence of mercury has been demonstrated here to be a important feature of mercury-resistant cells for the application in bioremediation processes.

The 16S rRNA gene sequence analysis revealed that ten mercury-resistant bacterial strains were identified as *Pseudomonas* spp., *Stenotrophomonas* sp., and *Aeromonas* sp. (Table 4). To date, the genera *Pseudomonas*, *Stenotrophomonas* and *Aeromonas* comprise 198, 13 and 30 species, respectively, with validly published names, at the time of writing (List of Prokaryotic Names with Standing in Nomenclature (LPSN); http://www.bacterio.cict.fr/). Of the ten bacterial strains, eight strains were identified as *Pseudomonas* that was predominant within the Hg-resistant bacterial diversity in the sites (Table 4). The dominance of the genus *Pseudomonas* is not surprising, since the genus *Pseudomonas* has been recognized for its ability to utilize a broad spectrum of environmental pollutants such as organics and heavy metals (Tvrzova *et al*. 2006). The genus *Pseudomonas* is also known as a large and widely diverse bacterial group and members of the genus *Pseudomonas* are ubiquitous in a wide variety of habitats such as soil, water and sediments (Young and Park 2007). Of the members of the genus *Pseudomonas*, *Pseudomonas plecoglossicida* predominated in all samples (sediments III and IV) from which five strains of *P. plecoglossicida* were successfully isolated (Table 4). The species *P. plecoglossicida* has been reported to be a causative agent of fish disease (Nishimori *et al*. 2000). Two other *Pseudomonas* species were also obtained in this study; two strains of the species *Pseudomonas hibiscicola* from sediment IV sample and one strain of the species

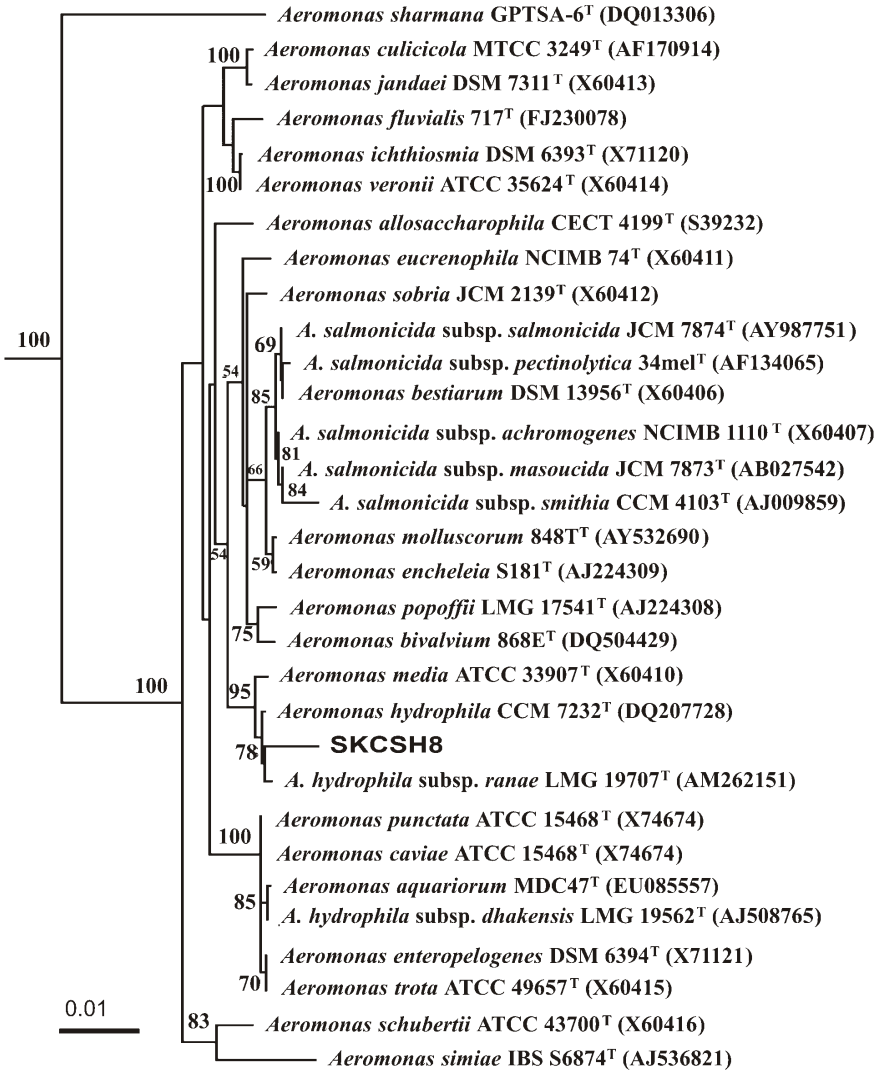

Fig 3 Phylogenetic tree of strain SKCSH8, based on 16S rRNA gene sequences. The branching pattern was produced by the neighbour-joining method. Numbers at nodes are percentage bootstrap values based on 1000 iterations; only values above 50% are shown. GenBank accession numbers are given in parentheses. Bar, 1 substitutions per 100 nucleotides.

Pseudomonas moraviensis from tailing soil sample (Table 4). The first strain of the species *P. moraviensis* was isolated from soil contaminated with nitroaromatis in Moravia, in the Czech Republic (Tvrzova *et al*. 2006).

In addition to the genus *Pseudomonas*, one species of the genus *Aeromonas* (i.e., *Aeromonas hydrophila* subsp. *ranae*) was also recovered from sediment II sample (Table 4). Members of the genus *Aeromonas* have been recognized as opportunistic human pathogens, and strains of *Aeromonas hydrophila* are also pathogenic to amphibians (Huys *et al*. 2003). They are also widely distributed in freshwater environments (Holmes *et al*. 1996) and some strains of *Aeromonas hydrophila* are reported to be resistant to heavy metals (Miranda and Castillo 1998). Apart from the genera *Pseudomonas* and *Aeromonas*, one member of the genus *Stenotrophomonas* (i.e., *Stenotrophomonas africana*) was present in the sediment II sample (Table 4). The genus *Stenotrophomonas* was created in 1993 to accommodate *Xanthomonas maltophila* (formerly *Pseudomonas maltophila*) (Palleroni and Bradbury 1993), and *Stenotrophomonas africana* is a later synonym of *Stenotrophomonas maltophila* (Coenye *et al*. 2004). The species *S. africana* is an essensial cause of nosocomial infection that is present in a wide range of environmental niches (Drancourt *et al*. 1997). The genus *Stenotrophomonas* is frequently found in soils and especially in the plant rhizospheres which are particularly contaminated with potentially toxic metals such as copper, platinum, mercury, gold, cadmium, lead, chromium, silver, and selenium salts (Barkay *et al*. 2003).

Although large numbers of the genus *Pseudomonas* are capable of being resistant to mercury, they are not representatives of the type strains of mercury-resistant

bacteria. Until presently, there are only two type strains of mercury-resistant bacteria in the world which were *Alteromonas tagae* sp. nov. (type strain AT1^T = BCRC 17571^T-JCM 13895^T) and *Alteromonas simiduii* sp. nov. (type strain AS1^T = BCRC 17572^T – JCM 13896^T) isolated from water samples of the Er-Jen River estuary, Tainan, Taiwan (Chiu *et al*. 2007). Thus, our findings of Hg-resistant bacteria (*Pseudomonas moraviensis* strain SKCSH1 and *Pseudomonas plecogossicida* strains SKCSH4, SKCSH7, and SKCSH9) could be regarded as the type strains of four novel species in the genus *Pseudomonas* that will add to the diversity of Hg-resistant bacteria worldwide. The distinct nature of their phylogenetic position suggests that these four isolates have the high possibility to be novel species of the genus *Pseudomonas* due to their 16S rRNA gene sequence similarities below 97% (the threshold recognized as delineating a genospecies (Stackebrandt and Goebel 1994; Prakash *et al*. 2007; Tindall *et al*. 2010)).

In conclusion, the present study has provided the first evidence of the phylogenetically-diverse Hg-resistant bacteria in the Hg-polluted sites of Indonesia in which the genus *Pseudomonas* predominates. Likewise, the bacteria are highly resistant to Hg concentrations ($HgCl_2$) of 60-550 mg L^{-1}. Such information may prove highly useful for developing in situ bioremediation of Hg-contaminated sites in Indonesia.

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