Indigenous mercury-resistant bacteria isolated from contaminated soils around artisanal gold processing centers in Sukabumi, Indonesia

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Abstract. In Indonesia, the largest mercury pollution comes from artisanal and small-scale gold mining (ASGM), which may cause the distribution of mercury to agricultural land and can be absorbed by food crops. Sukabumi Regency in West Java, well-known as one hotspot of illegal artisanal gold mining and national rice producer, is potentially threatened by mercury pollution. Efforts to remediate mercury contaminated agricultural land can be done by using mercury-reducing bacteria. This research aims to select the most potential indigenous bacteria for mercury remediation. Soil and sludge samples were collected from 2 districts in Sukabumi, where gold processing using mercury is common. Bacteria were selectively isolated from cultured colonies grown in Luria Bertani broth supplemented with HgCl2 30 mg/L. We obtained 27 isolates that belong to 16 species, as identified by API® 20 E and 20 NE (BioMérieux, USA). The growth of each isolate was assessed by measuring the optical density of inoculated LB broth contained HgCl2 30 mg/L for 5 consecutive days. All isolates showed normal growth. The log phase reached its maximum value on the second or third day after inoculation and lag phase afterward. Twelve identified isolates were chosen for evaluation of their resistance to mercury by growing them in Mueller-Hinton agar supplemented with HgCl2 (30 mg/L, 50 mg/L, 100 mg/L, 150 mg/L, and 200 mg/L). Seven isolates were able to grow in media with HgCl2, but only Mer07 survived on HgCl2 150 mg/L.

1. Introduction
Mercury is a well-known heavy metal for its toxicity causing public health disasters in Minamata, Japan. Mercury and its compounds are very dangerous to humans and the environment because they are toxic, persistent, bioaccumulated in various media, and can be transported over long distances through the atmosphere [1]. Meanwhile, concentrations of global mercury have increased approximately threefold due to various anthropogenic activities [2].

In Indonesia, artisanal and small-scale gold mining (ASGM) is the most significant contributor to mercury emissions [3]. ASGM activities often pollute soil, water, and air, which can be transmitted widely and contaminate the surrounding biota. Many studies have reported mercury contamination on agricultural soils around ASGM areas in Indonesia [4–6]. As a result, land productivity decreases, agricultural and fishery products become contaminated with mercury, and public health is threatened [7–9].
A human can be exposed to mercury through inhalation as elemental vapor and through digestion [10] by consuming water or food coming from contaminated lands. A study on Mount Botak, Buru Island, Maluku [4] showed that mercury contamination exceeds the 50 μg/kg threshold in food crops grown on land near illegal gold mining. Likewise, rice plants in Lebaksitu, Banten, are reported to contain higher mercury levels when the rice fields’ location is closer to the ASGM area [5].

A study by [11] shows that mercury exposure to the people who live nearby gold processing facilities in Sukabumi regency, West Java was the majority in alert level. This indicates that the mercury pollution in this area is already alarming. There is a high possibility that the mercury has been spreading to the agricultural lands around the gold processing facilities.

To overcome mercury contamination in agricultural land, it is necessary to carry out effective remediation to prevent mercury from being absorbed into crops. Remediation techniques, both mechanical and chemical, have disadvantages in terms of high costs and by-products produced. On the other hand, remediation using biological elements, so-called bioremediation, has been proven as an environmentally friendly, effective, and economical technology for restoring contaminated soil [12].

In principle, bioremediation utilizes plants, bacteria, or fungi to reduce pollutants released into the ecosystem or make them less or non-toxic. Bioremediation can be performed by stimulating the growth of local microorganisms, called biostimulation, or by utilizing other microorganisms with the ability to remediate pollutants [13].

Microorganisms such as bacteria are suitable as bioremediation agents because they can live and reproduce in extreme conditions [14]. Moreover, bacteria are a resource that is abundantly available in nature even in areas contaminated with harmful pollutants such as heavy metals [15,16]. There have been many studies on the use of microorganisms to remediate heavy metals [17–19]. Bioremediation of mercury alone has been reported using different strains of bacteria [20–22].

The present work was aimed to isolate mercury-resistant bacteria from contaminated soils taken from gold processing centers in Sukabumi regency, West Java, and determine the best isolates based on the mercury resistance test for further investigation on the potential of these isolates as mercury bioremediation agents.

2. Method
2.1. Study site
Sukabumi Regency is well-known as one hotspot of illegal artisanal gold mining, and as the biggest regency in West Java, it is also one of the national paddy producers. At least 50 spots of unlawful artisanal gold mining are reported in Sukabumi, mostly concentrated in the southern part of the regency [23]. Due to recent illegal mining repression conducted by the local authority, the activities including gold processing were terminated, though few were still covertly operating. Sampling sites were selected in six traditional gold processing facilities, so-called gelundung, that were still operating at the moment of data collection, located in Langkap Jaya village (Lengkong district), Waluran Mandiri, and Mangun Jaya villages (Waluran district). The gold miners in Sukabumi have been using gelundung to detach the gold from other minerals by adding elemental liquid mercury to the mined rocks. Gelundung in Waluran and Lengkong districts mostly are located next to the owner’ house and surrounded by paddy rice fields or house gardens. A paddy field near to Ciletuh Geopark was taken as a control sample in which gold processing activities were not found as confirmed by the local authority.

2.2. Sample collection
Samples, comprised of rhizosphere soils, non-rhizosphere soils, and sludges, were collected during the dry season from the study site. Rhizosphere soil samples (sample ID: 1R – 6 R) were taken from the roots of plants (paddy, cassava, and papaya) that were cultivated in paddy fields or house gardens next to gelundung. Meanwhile, non-rhizosphere soil samples (sample ID: 1NR – 6 NR) were collected from nine different points around the gelundung to make a composite following [24]. Sludge samples (sample ID: 1S – 6 S) were taken from the tailing pond next to the gelundung. All samples were collected by clean handling to prevent cross-contamination and directly stored in cooler boxes while being transported to the laboratory and kept at about 4°C before analysis.
Figure 1. Six sampling sites in Waluran district (sampling sites no. 1-3) and Waluran district (sampling sites no. 4-6) of Sukabumi regency.

2.3. Bacteria isolation and identification
Prior to the bacteria isolation, 10 mL of diluted soil/sludge samples were inoculated into 100 mL Luria Bertani (LB) broth supplemented with 0.3 mg HgCl$_2$ and without HgCl$_2$ as control. After 24 hours incubation at 37°C, the inoculation products were evenly spread on petri dishes filled with the Plate Count Agar (PCA) and then kept at 37°C at least overnight until a number of bacteria colonies were visible. Macroscopic observation was performed to determine the colonies’ characteristics, such as color, shape, elevation, and colony margin following [25]. Based on those characteristics, different colonies were separately isolated by spreading technique on PCA-filled petri dishes. After 24 hours, the isolated cultures were propagated in LB broth + HgCl$_2$ 30 mg/L to observe the growth of the bacteria. The growth curve was determined by measuring the optical density of the culture in triplicate using a Shimadzu UV1601 spectrophotometer (Shimadzu, Japan) with the wavelength of 600 nm [25] every 24 hours for 5 consecutive days. Each of isolated bacterium was pre-identified by performing Gram-staining [26] and oxidase test [27], and further identification using API® 20E or 20NE (BioMérieux, USA) following the manufacturer’s guideline.
2.4. Hg-resistant test
The mercury resistance for each bacterial isolate was assessed by measuring the Minimum Inhibitory Concentration (MIC) of HgCl$_2$ which able to suppress the growth of the bacteria inoculated in Mueller-Hinton agar media. Each isolate was grown in petri dishes filled with Mueller-Hinton media supplemented with HgCl$_2$ with the concentration of 0 mg/L, 30 mg/L, 50 mg/L, and 100 mg/L. After incubation at 37°C for 24 hours, the intensities of colonies grown in media with HgCl$_2$ (30 mg/L, 50 mg/L, and 100 mg/L) were compared to the one without HgCl$_2$ (blank).

3. Results and discussion
3.1. Isolation of mercury-resistant bacteria
Bacteria were isolated from soil samples (rhizosphere and non-rhizosphere soils) and sludge samples as much as 20 samples. The density of bacterial colonies grown in LB media with and without the supplementation of HgCl$_2$ is as shown in Table 1. The total mercury concentration of each sample was derived from a study by [29]. Bacteria from the sludge samples taken from Plot 2 (sample ID 2S) and Plot 4 (sample ID 4S) did not grow at all when inoculated into LB media with 30 mg/L HgCl$_2$ added. Meanwhile, the highest colony density was found in the petri dish from the rhizosphere soil sample taken from Plot 4 (sample ID 4R) as much as 105 x 10$^9$ colonies per mL.

| Sample ID | Total mercury concentration (mg/L) | Number of colonies (x 10$^5$ per mL) |
|-----------|-----------------------------------|-------------------------------------|
| 1R        | <30                               | 17.300                               |
| 1NR       | 30-100                             | 14.300                               |
| 1S        | >100                              | 183                                 |
| 2R        | <30                               | 6.800                                 |
| 2NR       | <30                               | 10.300                               |
| 2S        | >100                              | 123                                 |
| 3R        | 30-100                             | 8.300                                 |
| 3NR       | 30-100                             | 216.000                               |
| 3S        | 30-100                             | 9.000                                 |
| 4R        | <30                               | 266.000                               |
| 4NR       | <30                               | 290.000                               |
| 4S        | 30-100                             | 380                                  |
| 5R        | <30                               | 301.000                               |
| 5NR       | <30                               | 11.700                               |
| 5S        | >100                              | 21.200                               |
| 6R        | <30                               | 15.400                               |
| 6NR       | <30                               | 15.000                               |
| 6S        | >100                              | 28.500                               |
| Control R | <30                               | 6.800                                 |
| Control NR| <30                               | 970                                   |

Note: R: rhizosphere soil sample, NR: non-rhizosphere soil sample, S: sludge sample

The number of colonies that grew in the media without HgCl$_2$ was not always higher than those in the media with HgCl$_2$, thus indicating that the presence of HgCl$_2$ might be beneficial for the growth of certain bacteria. Furthermore, there is no clear correlation between the total mercury concentration of the samples from which the bacteria were derived and the colony density of the bacteria grew, either in the media with HgCl$_2$ or without HgCl$_2$. According to [30], some bacteria can adapt to growth in the
presence of mercury more rapidly than others. A study by [31] reports that the presence of the pollutant is one of the factors that influence the adaptability of the bacteria to the media. Furthermore, according to [32], bacteria have developed resistance to various toxic metals to survive in a polluted environment.

The isolation of each distinguished colony resulted in a total of 27 bacterial isolates shown in Table 2. The majority of these isolates are white pigmented with round shapes, flat edges, and convex or flat elevations.

Table 2. Morphological characteristics of 27 isolated bacterial colonies.

| Isolate ID | Pigmentation | Shape | Margin | Elevation |
|------------|--------------|-------|--------|-----------|
| Mer01      | White        | Rod   | Undulate | Flat     |
| Mer02      | White        | Rod   | Undulate | Flat     |
| Mer03      | White        | Rod   | Entire  | Raised   |
| Mer04      | White        | Rod   | Entire  | Raised   |
| Mer05      | White        | Rod   | Entire  | Raised   |
| Mer06      | White        | Rod   | Entire  | Raised   |
| Mer07      | White        | Rod   | Undulate | Flat     |
| Mer08      | White        | Rod   | Entire  | Raised   |
| Mer09      | White        | Rod   | Entire  | Raised   |
| Mer10      | White        | Rod   | Entire  | Raised   |
| Mer11      | White        | Rod   | Undulate | Flat     |
| Mer12      | White        | Rod   | Entire  | Raised   |
| Mer13      | White        | Rod   | Entire  | Flat     |
| Mer14      | White        | Rod   | Entire  | Flat     |
| Mer15      | White        | Rod   | Entire  | Raised   |
| Mer16      | White        | Rod   | Entire  | Raised   |
| Mer17      | White        | Rod   | Entire  | Raised   |
| Mer18      | White        | Rod   | Entire  | Raised   |
| Mer19      | White        | Rod   | Entire  | Raised   |
| Mer20      | White        | Rod   | Entire  | Raised   |
| Mer21      | White        | Rod   | Entire  | Flat     |
| Mer22      | White        | Rod   | Entire  | Raised   |
| Mer23      | White        | Rod   | Entire  | Flat     |
| Mer24      | White        | Rod   | Undulate | Flat     |
| Mer25      | White        | Rod   | Undulate | Flat     |
| Mer26      | White        | Rod   | Entire  | Flat     |
| Mer27      | White        | Rod   | Entire  | Flat     |

3.2. Calculation of growth curve

The adaptability of investigated bacteria to the mercury-contaminated environment was tested by inoculating the isolate in LB broth supplemented with HgCl₂ 30 mg/L. The growth of the respective bacteria in such conditions is indicated by the increase of optical density of the broth through five days of observation. The results of the optical density measurement of each isolate are depicted as a growth curve shown in Figure 2.

The curve was calculated as the mean of optical density from 27 isolates, starting from the incubation day (H0) to the fifth day (H5). The highest growth rate occurred between H0 to H2, and the growth was pretty much flat afterward. This indicates that the isolates were able to grow normally with the presence of HgCl₂ 30 mg/L. Several studies [20, 33–36] show a similar pattern the growth curve of different bacteria in mercury-supplemented media.
3.3. Bacteria identification

Based on the results of Gram staining, it is known that all isolates obtained in this study are Gram-negative bacteria. Gram-negative bacteria are protected by a thin peptidoglycan cell wall surrounded by an outer membrane containing lipopolysaccharide [37]. Compared to the Gram-positive bacteria, which is surrounded by a thick peptidoglycan cell wall, the cell envelopes of Gram-negative bacteria are composed of three structural entities, namely an inner or cytoplasmic membrane, a thin and rigid cell wall, and an outer membrane [38]. According to [39], the Gram-negative bacteria is resistant to heavy metal due to the presence of lipopolysaccharide structure in their cell wall. Moreover, studies by [40, 41] showed that Gram-negative bacteria tend to be more tolerant to heavy metals than the Gram-positive.

To identify the species of Gram-negative bacteria, API® 20E and 20NE (BioMérieux, USA) were used. As many as 21 isolates were identified with a suitability level above 70% using API® 20E, only one isolate (Mer20) was identified using API® 20NE. Furthermore, two isolates (Mer11 and Mer12) were identified with a low level of conformity, and three isolates (Mer02, Mer03, Mer06) could not be identified at all either with API® 20E or API® 20NE.

Table 3 shows 13 different species of bacteria identified in this study. Seven of them belong to the family of Enterobacteriaceae, namely Enterobacter cloacae, Cedecea lapagei, Enterobacter asburiae, Klebsiella pneumoniae, Cronobacter spp., Shigella spp., and Citrobacter freundii, and the rest belong to family Aeromonadaceae (Aeromonas salmonicida), Flavobacteriaceae (Elizabethkingia meningoseptica), Pseudomonaceae (Pseudomonas oryzihabitans, Pseudomonas fluorescens/putida), Shewanellaceae (Shewanella putrefaciens), and Vibrionaceae (Vibrio cholerae).

The variation of bacteria that were found in the mercury-contaminated soils in this study indicates that bioremediation agents are abundantly available in nature. According to [42], the abundance of heavy metal resistant bacteria in nature helps the destruction or transformation of toxic substances that pollute the environment.

Most of the reported species in Table 3 have a promising potential as bioremediation agents for environmental pollutant heavy metals, such as lead (Pb), cadmium (Cd), chromium (Cr), nickel (Ni), zinc (Zn), copper (Cu) and including mercury (Hg). At least nine species of bacteria isolated in this study were reported to be tolerant to mercury, meanwhile one species (Elizabethkingia meningoseptica) was so far never been reported to be resistant to any particular heavy metal.
Table 3. Result of bacteria identification and relevant studies regarding the heavy metal resistance of each bacteria species.

| Isolate ID | Identification result | Family | Resistance to heavy metal |
|------------|-----------------------|--------|---------------------------|
| Mer01      | *Aeromonas salmonicida*<sup>a</sup> | Aeromonadaceae | Hg [43] |
| Mer04, Mer18, Mer26 | *Enterobacter cloacae*<sup>a</sup> | Enterobacteriaceae | Pb, Cd, Cr, Hg dan Cu [44] |
| Mer05      | *Cedecea lapagei*<sup>b</sup> | Enterobacteriaceae | Intolerant to Cd, Cu, Cr, Ni, Pb, Co, Zn [43] |
| Mer07      | *Elizabethkingia meningoseptica*<sup>b</sup> | Flavobacteriaceae | No report found |
| Mer08, Mer15, Mer19, Mer27 | *Enterobacter asburiae*<sup>a</sup> | Enterobacteriaceae | Methylmercury [44] |
| Mer09      | *Pseudomonas oryzihabitans*<sup>b</sup> | Pseudomonaceae | Cu, Zn [45] |
| Mer10, Mer17 | *Pseudomonas fluorescens/putida*<sup>b</sup> | Pseudomonaceae | Hg [36, 46] |
| Mer13, Mer14 | *Klebsiella pneumoniae*<sup>a</sup> | Enterobacteriaceae | Li, Cd, Hg [47] |
| Mer16, Mer21 | *Cronobacter spp.*<sup>a</sup> | Enterobacteriaceae | Hg [48] |
| Mer20      | *Shewanella putrefaciens*<sup>a</sup> | Shewanellaceae | Hg [49] |
| Mer22      | *Shigella spp.*<sup>a</sup> | Enterobacteriaceae | Tolerant to Ag, Zn, Cu, Pb with low concentration [50] |
| Mer23      | *Vibrio cholerae*<sup>a</sup> | Vibrionaceae | Hg, Zn, Pb, Cd, Cr, Ni [51] |
| Mer24, Mer25 | *Citrobacter freundii*<sup>a</sup> | Enterobacteriaceae | Hg [52] |

Note: <sup>a</sup>Facultative anaerobe,<sup>b</sup>Obligate aerobe

Amongst the identified isolates, three bacteria are widely known as pathogens, namely *Vibrio cholerae* [53], *Klebsiella pneumoniae* [54], and *Elizabethkingia meningoseptica* [55]. However, cautious handlings were performed especially to these pathogens to prevent disease infection.

3.4. Mercury resistance test

In this testing, one isolate was taken to represent each species. *Pseudomonas oryzihabitans* was excluded from the testing since the only isolate (Mer09) was unable to be regrown in PCA agar media. The isolate with the highest density of regrown colony in PCA agar was chosen to represent species with an isolated number of more than one.

The HgCl₂ concentration used for the mercury resistance test in this study were 30, 50, 100, 150, and 200 mg/L. Several studies [56, 57] used a concentration range between 0 to 200 mg/L to measure the minimum inhibitory concentration of mercury. The results of mercury resistance test for each species are shown in Table 4.

The quantification of resistance level was done by comparing the density of the colony grew in media supplemented with and without HgCl₂. When the colony grew in media with HgCl₂ was at least 75% as dense as the one in media without HgCl₂, it was valued as +++++, 50-75% was valued ++++, 25-50% was valued ++, <25% was valued +, and when there was no colony grew at all in the media with HgCl₂, it indicated the isolate was not resistance to the respective concentration of HgCl₂.
Table 4. Mercury-resistance test result of 12 identified bacteria.

| Isolate ID | HgCl₂ 30 mg/L | HgCl₂ 50 mg/L | HgCl₂ 100 mg/L | HgCl₂ 150 mg/L | HgCl₂ 200 mg/L |
|------------|---------------|---------------|----------------|----------------|---------------|
| Mer01      | +++           | +++           | ++             |                |               |
| Mer05      | ++            | ++            | -              |                |               |
| Mer07      | ++            | +++           | +++            | -              | -             |
| Mer13      | +++           | ++            | -              |                |               |
| Mer17      | +++           | -             | -              |                |               |
| Mer18      | +++           | +++           | +++            | -              | -             |
| Mer19      | +++           | +++           | +++            | -              | -             |
| Mer20      | +++           | +++           | +++            | -              | -             |
| Mer21      | +             | +++           | -              |                |               |
| Mer22      | +++           | +++           | +++            | -              | -             |
| Mer23      | +++           | +++           | +++            | +++            | -             |
| Mer25      | +++           | ++            | -              |                |               |

Note: Isolates other than Mer07, Mer19, and Mer23 were not included in the resistance test with HgCl₂ >100 mg/L. The resistant values were quantified by comparing the intensity of colony growth in media with and without HgCl₂. “-” means no growth observed, “+” means the growth is <25%, “++” means the growth is 25-50%, “+++” means the growth is 50-75%, and “++++” means the growth is >75%.

This resistance test showed that all the investigated isolates were able to grow in media with HgCl₂ 30 mg/L, which was as expected as isolation of these bacteria was done selectively by adding HgCl₂ 30 mg/L into LB broth. When the concentration of HgCl₂ was increased up to 50 mg/L, three isolates (Mer01, Mer13, and Mer25) were growing less and the other three were growing more (Mer07, Mer21, and Mer23). When the concentration of HgCl₂ was increased up to 100 mg/L, five isolates (Mer05, Mer13, Mer17, Mer21, and Mer25) were unable to propagate.

Table 5. Mercury resistance in different bacteria reported by several studies.

| Species Name | HgCl₂ (mg/L) | Citation |
|--------------|--------------|----------|
| *Bacillus thuringiensis, E. coli* | 50 | [20] |
| *Morganella morganii* | 130 | [58] |
| *Pseudomonas aeruginosa, P. otitidis,* *P. stutzeri, P. mendocina, Klebsiella pneumonia, Bacillus sp.* | 160 | [59] |
| *Stenotrophomonas maltophilia* | 250 | [19] |
| *Vibrio fluvialis* | 550 | [60] |

Several studies reported that higher pollutant concentration contained in the media resulted in lower bacteria growth [61, 62]. Contradictions were found in this study, such as Mer07 and Mer23 were performed better in HgCl₂ 50 mg/L and 100 mg/L, and Mer21 was growing much better in media with HgCl₂ 50 mg/L compared to HgCl₂ 30 mg/L but did not grow at all with HgCl₂ 100 mg/L. However, a similar observation was recorded by [63] that the response of some bacteria grown in diesel-contaminated media was not always correlated to the increase of the pollutant concentration. Furthermore as shown in Table 5, several studies [19, 20, 58–60] report on the resistance of different bacteria to mercury, in particular *Bacillus thuringiensis* and *E. coli* survived in the media with HgCl₂ up to 50 mg/L of HgCl₂, meanwhile the growth of *Aeromonas hydrophila* was inhibited by the presence of 550 mg/L of HgCl₂.
Figure 3. The colony appearance of isolate Mer07, Mer19, and Mer23 in MH media supplemented with HgCl$_2$ 50 mg/L, 100 mg/L, and 150 mg/L.

4. Conclusion
The study successfully isolated thirteen Gram-negative bacteria species from mercury-contaminated soil samples and tested their resistance to HgCl$_2$ up to 200 mg/L. The result indicated that mercury-resistant bacteria are indigenously available in the contaminated site. All the isolated species were able to grow normally in media with the presence of HgCl$_2$ 30 mg/L. These organisms can be used in the future to explore the most applicable and cost-efficient methods for mercury bioremediation. Furthermore, the use of bacterial consortia for mercury degradation is still less investigated. Thus in-depth research on this matter is recommended.

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