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Identification of mercury-resistant *Streptomyces* isolated from *Cyperus rotundus* L. rhizosphere and molecular cloning of mercury (II) reductase gene

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ABSTRACT *Streptomyces* is one of mercury-resistant bacteria which can convert Hg^{2+} into nontoxic Hg^{0}. This study aimed to identify mercury-resistant *Streptomyces* present in the *Cyperus rotundus* rhizosphere from artisanal small-scale gold mining (ASGM) area and clone *merA* gene to the cloning and expression vectors. Molecular identification was conducted using 16s rRNA gene with the maximum likelihood algorithms. Results revealed that the AS1 and AS2 strains were a group of *Streptomyces ardesiacus* and the BR28 strain was closed to *Brevibacillus agri*. The AS2 *merA* gene was cloned to pMD20 cloning vectors, pGEX-5x-1 and pET-28c expression vectors. The transformation was successfully performed in BL21 and DH5α competent cells. The full length of the *merA* gene was confirmed to be 1,425 bp. This study is the first research on identifying mercury-resistant *Streptomyces* and cloning the full-length *merA* gene in Indonesia.

KEYWORDS artisanal gold mining, mercuric reductase, 16s rRNA, plasmid vector

1. Introduction

Mercury contamination commonly occurs in the artisanal gold mining areas in Indonesia due to gold amalgamation (Krisnayanti et al. 2012). This contamination is a serious environmental problem that adversely affects human health, especially that of gold mining workers (Niane et al. 2019). Chemical and physical methods for removing heavy metals have been studied for many years, but these methods have created new problems, such as the generation of chemical waste, complicated post-processing, and wasteful costs (Alotaibi et al. 2021). Bioremediation strategies using plants (phytoremediation) and microorganisms have been developed as attractive alternatives because they are effective, inexpensive, and efficient (Tiodar et al. 2021).

Streptomyces are fungi-like appearance bacteria, usually living on the soil or plant rhizosphere. *Streptomyces* produce important metabolites such as antibacterial, special enzyme and protein. However, reportedly mercury-resistant bacteria were *Firmicutes, Planctomycetes, Bacillus, Gammaproteobacteria, Brevundimonas, Nitrococcus, Fusobacterium, Stenotrophomonas, Arthrobacter, Bacillus and Pseudomonas* (Purkan et al. 2017; Chasanah et al. 2018; Niane et al. 2019; Fatimawali et al. 2020; Singh and Kumar 2020). On the other hand, *Streptomyces* was a potential bacteria on mercury transformation due to producing high activity of mercuric reductase enzyme, but rarely explored (Rahayu et al. 2021).

Streptomyces was reported to reduce mercury ions (Hg^{1+}, Hg^{2+}) into elemental mercury (Hg°) because of their mer operon system located in the linear plasmid (Boyd and Barkay 2012), genomic DNA (Xiao-xi et al. 2010), and Tn21 transposons or integrons (Liebert et al. 1999). The mer operon is a positive type of operon that

is immensely conserved. It consists of the operator gene; promoter gene; regulatory gene (*merR*); and functional genes, including *merT*, *merP*, *merD*, *merF*, *merC*, *merA*, and *merB* in broad range of operons (Singh and Kumar 2020). These genes code for a particular protein and execute a particular function in mercury-resistant bacteria.

The cloning of the *merA* gene from *Streptomyces coelicolor* M130 was first reported by Hopwood (Hopwood 1983). This technology will presumably reduce mercury contaminants in the soil through cell bioaugmentation processes. Cell bioaugmentation is based on the survival and catabolic activity of inoculated microbial strains (Mosa et al. 2016). The inoculation of bacteria harboring the necessary metabolic pathways for the degradation of target contaminants accelerate the removal of contaminants and reduces the time required for the intended bioremediation (Tiodar et al. 2021).

A previous study explored *Streptomyces* in the rhizosphere of nutgrass around a traditional small-scale gold mining area in Lombok, Indonesia (Rahayu et al. 2021). In that study, *Streptomyces* spp. were isolated, and four isolates were categorized as mercuric-resistant strains (Rahayu et al. 2021). However, studies on identifying mercury-resistant *Streptomyces* and genes involved in mercury bioremediation are still limited, especially in Indonesia. This research was performed to identify mercury-resistant *Streptomyces* and clone the mercury reductase gene in pMD20 cloning vector, pGEX-5x-1 and pET-28c expression vectors.

2. Materials and Methods

2.1. Subculture of Streptomyces

Mercury-resistant *Streptomyces* isolates of strains AS1, AS2, and BR28 from *Cyperus rotundus* rhizosphere around traditional gold mining in Selodong Hamlet, Buwun Mas Village, Sekotong District, West Lombok, Indonesia (Rahayu et al. 2021) were grown in a yeast extract malt agar (YEMA) with nystatin. The grown isolates were purified using starch nitrate agar (SNA). The isolates obtained were then cultured in slanted SNA and stored at room temperature for the next experiments.

2.2. Mercury resistance ability test

The Mercury resistance test was conducted using the paper disk method. Exactly 0.1 mL of spore suspension was inoculated in SNA using the surface plate method. As much 2, 4, and 6 µL of $HgCl_2$ I mM was put on the 6 mm diameter paper disk in the SNA medium, incubated at room temperature for seven days. Strain with less than 1 cm clear zone diameter was categorized as a resistant strain.

2.3. Genome isolation and identification based on 16s rRNA gene

Chromosomal DNA isolation was performed using the spooling with a glass rod according to the method (Hopwood 1983) with modifications. The isolation of the 16s rRNA gene was performed using the PCR method with general primers 16sF (EC F 5' TCTGCAGTCGACGATGACCAC) and 16sR (EC R3' GTGCCAGCAGCCGCGGTAATA). This gene was amplified using 0.5 µL Ex Taq, 25 µL 2× buffer I, 8 µL dNTP mix, 1 µL (10 ng) DNA template, 0.5 µL Primer F (100 pmol/µL), 0.5 µL Primer R (100 pmol/µL), and water until the volume reached 50 µL (100 pmol/µL). The PCR program was run at 94 °C for 1 min, (94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min) for 30 cycles, and 72 °C for 5 min. The PCR products were purified using the column spin method (Geneaid and MD Bio) and sequenced using the PT. Genetika Sain service.

The 16S rRNA sequences were aligned using ClustalW software (Thompson et al. 1994). Type strains references were downloaded from www.ezbiocloud.net based on preliminary checking. The evolutionary trees for the datasets were inferred from the maximum likelihood method based on the Tamura-Nei model using MEGA X (Kumar et al. 2018). The initial tree(s) for the heuristic search were obtained automatically by applying the neighbor-joining and BioNJ algorithms. The topology with a superior log likelihood value was finally selected with the 1,000 resamplings (bootstrap values).

2.4. Isolation and purification of merA gene

The *merA* primer was constructed by ClustalW analysis. The gene isolation was performed by PCR using a specific primer (Table 1). The PCR mixture contained 0.5 µL LA Taq, 25 µL 2× GC buffer I, 8 µL dNTP mix, 1 µL (10 ng) DNA template, 0.5 µL Primer F (100 pmol/µL), 0.5 µL Primer R (100 pmol/µL), water until a volume of 50 µL was reached (100 pmol/µL). The PCR program was run at 94 °C for 1 min, (94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min) for 25 cycles, and 72 °C for 5 min. Purification was conducted by the glass powder method (Marko et al. 1982).

2.5. Cloning to the vector

The cloning vector used in this work was pMD20. The ligation of *merA* gene to the cloning vector was performed by mixing 3 µL 10× T4 ligase buffer, 3 µL 10 mM ATP, 0.6 µL T-vector pMD20 (2.7 kB, 30 ng), 3 µL insert (*merA* gene 1.4 kB, 45 ng), 0.5 µL T4 ligase, and water up to 30 µL. The mixture was incubated at 16 °C overnight.

The expression vectors used in this work were pGEX-5x-1 and pET-28c. The mixture contained 10× buffer, 3 µg DNA, 10 U/µL restriction enzyme, and water. The mixture was then incubated at 37 °C for one h and subjected to agarose gel electrophoresis. DNA was purified using the glass powder method. The C-terminal, N-terminal, and vector were ligated. The ligation mixture contained 3 µL 10× ligase buffer, 3 µL 10 mM ATP, 1 µL vector (30 ng/µL), 3 µL N-terminal gene (2 ng/µL), 4 µL C-terminal gene (5 ng/µL), 0.5 µL T4 ligase, and water up to 30 µL. The mixture was incubated at 16 °C overnight and transformed into competent cells BL21.

TABLE 1 Sequences of merA primer

Primer	Sequence	Strain	Resource
merAF1-BamH1	ATA /GGA TCC/ CCG GTT ACG ATC TGG CG	AS1 and AS2	ClustalW analysis
merAR1-Xho1	ATA /CTC GAG/ TCA GCC GGC GCA GCA GGA	AS1 and AS2	ClustalW analysis
StrMerA-F2	ACT ACC TGA CCT CCA CCA G	AS1 and AS2	ClustalW analysis
Act-Fw	CGG ACT TCG TST ACG TCG C	BR28	(Oregaard and Sørensen 2007)
Act-Rv	GCC ATG AGG TAS GGG	BR28	(Oregaard and Sørensen 2007)
Slivid-5Up-F	ACT GAG GGA GGT AGT GCT	N-terminal of merA AS1, AS2	ClustalW analysis
AS2-RV-Pst1	TCA GCG CGA CCT GCA GGA CG	N-terminal of merA AS2	ClustalW analysis
AS1-RV NruI	CGG /TCG CGA/ TGA CGT AGT G	N-terminal of merA AS1	ClustalW analysis
AS2-Ini BamHI	TAT /GGA TCC/ TGC TCC AGG CAC ACA CC	N-terminal of merA AS1, AS2	ClustalW analysis

2.6. Transformation to E. coli BL21 and DH5α

As much as 3 µL of ligation mixture was transferred to 200 µL competent cells (BL21 and DH5α) by heat shock method (Anggoro and Ratnaningsih 2018). Thereafter, 100 µL of the mixture was placed in 900 µL LB agar with ampicillin (for pMD20 and pGEX-5x-1) and kanamycin (for pET-28c). Positive colonies were picked and inoculated into LB with kanamycin (for pET-28c) and ampicillin (for pMD20 and pGEX-5x-1). After overnight incubation at 37 °C, the plasmid was isolated using the alkaline lysis method.

2.7. Transformant confirmation and sequencing

The plasmid transformant was confirmed using the enzyme digestion method. As much as 2 µL of plasmid was added with the digestion mixture (1 µL 10× K buffer, 0.3 µL *Xho*I, 0.3 µL *Pst*I or *Bam*HI, and 6.4 µL water). The mixture was incubated at 37 °C for 1 h, added with 1 µL loading dye solution, and then subjected to agarose gel electrophoresis. The digestion enzyme was modified on the basis of the restriction site in each vector. Confirmation was also performed by sequencing procedures using BigDye version 1 or 3.

3. Results and Discussion

3.1. Mercury resistance ability test

Mercury-resistant *Streptomyces* isolates consisting of strains AS1, AS2, and BR28 from liquid glycerol medium and slant culture were grown back in a YEMA plate medium with nystatin to prevent contamination from mold. *Streptomyces* are members of the gram-positive group of bacteria that can only grow on a medium with a source of complex carbohydrates, such as SNA. The medium is acidified at a pH of 5 to maintain the solubility of mercury (Figure 1a). In this study, 1 mM of $HgCl_2$ was added to the filter paper. The clear zone less than 10 mm in the growth of *Streptomyces* on the SNA medium implied the resistance of *Streptomyces* to mercury (Figure 1b) (Table 2).

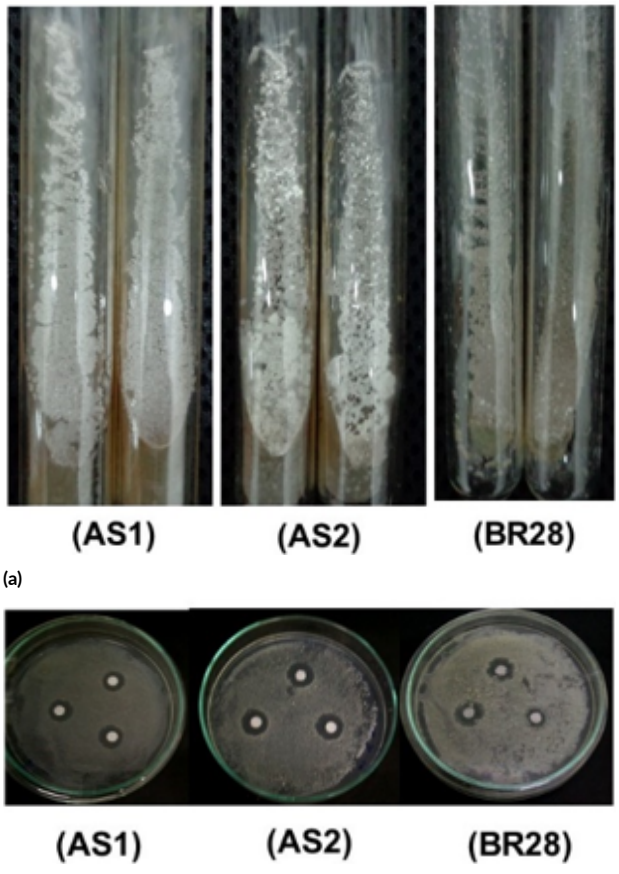

FIGURE 1 (a) Strain's colony in the SNA medium. (b) Mercury-resistant ability test using paper disk method.

3.2. Genome isolation and identification based on 16s rRNA gene

Chromosomal DNA isolation is shown in Figure 2a. This study successfully isolated the 16 s rRNA gene with a length of 1,504 bp by using a universal 16s primer (Figure 2b). As a molecular marker, 16s rRNA is the conserved gene that shows the phylogenetic relationship among prokaryotes. Herein, the sequences of these genes were compared with the 16s rRNA genes from GenBank.

TABLE 2 Diameter of clear zone

Isolates	Volume of 1 mM $HgCl_2$ (µL)		
	2	4	6
AS1	7.000±0.000	13.667±0.577	16.667±0.577
AS2	9.667±0.577	13.667±0.577	17.333±0.577
BR28	9.333±0.577	10.333±0.577	15.000±0.000

Diameter is in mm±SD

Replication was three times for each group

The complete sequences were analyzed and constructed using the phylogenetic analyzer of the MEGA X program (Kumar et al. 2001) (Figure 3). Based on the MCL analysis (Santorum et al. 2014) of the 16s rRNA gene, AS1 and AS2 were identified to be close to *S. ardesiacus*. This result was supported by the bootstrap value of 99% between AS1 and AS2 and *S. ardesiacus.* Surprisingly, BR28 was deemed close to *Brevibacillus agri*. The complete sequences of the 16S rRNA gene of strains AS1 and AS2 have been deposited in the DNA Data Bank of Japan (DDBJ), with accession numbers LC026159 and LC026160 for AS1 and AS2, respectively. The BR28 sequence is in the process of being submitted to GenBank.

The evolutionary history was inferred using the Maximum Likelihood method and Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with a superior log-likelihood value. This analysis involved 31 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1868 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

3.3. *merA* gene isolation

The *merA* gene was isolated from chromosomal DNA. However, obtained *merA* genes were not shown in specific bands (Figure 2c.); therefore, *merA* were purified using the glass powder method, checked with sequencing to gain the single band and correct *merA* full gene.

3.4. Cloning to the vectors

The cloning on the pMD20 vector results was confirmed by the restriction method. The *merA* gene showed a length of around 1,400 bp when the vector showed a length of 5.1 kbp. The complete sequences (1,425 bp) of the *merA* gene of AS2 have been deposited in the DDBJ with accession numbers LC026157 and LC026158. The *merA* gene of AS1 and BR28 has not been successfully sequenced yet.

The selected expression vector must be adjusted to the open reading frame of the cloned gene (*merA*). An error in the selection of expression vectors can cause changes

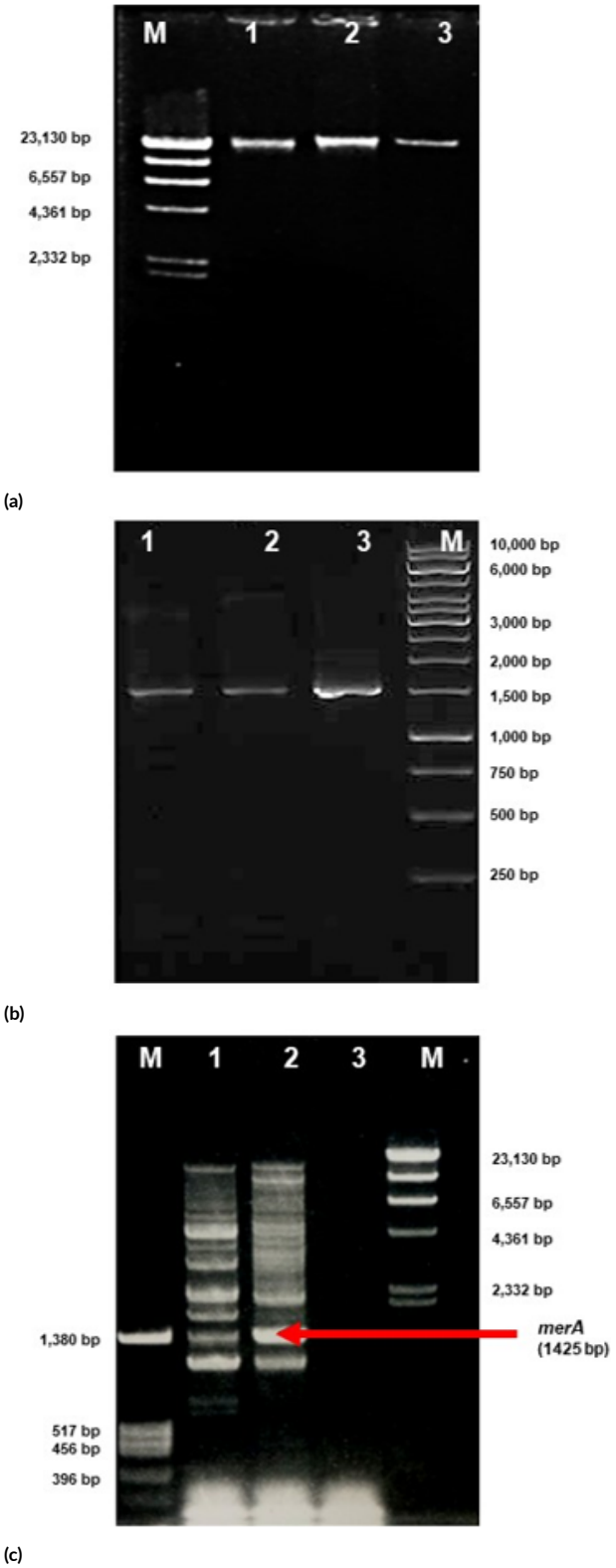

FIGURE 2 (a) Chromosomal DNA isolated using spooling with a glass rod, M: marker λ/*Hind*III, 1: *Streptomyces* isolate AS1, 2: *Streptomyces* isolate AS2, 3: *Streptomyces* isolate BR28. (b) 16s rRNA gene. (c) *merA* gene, M: marker BSM 13 and marker λ /*Hind*III.

in the open reading frame protein expressed. As shown in Figure 4a., the pET-28c vector cut with *Bam*HI and *Xho*I presents the correct cutting edge. Figure 4b. shows the

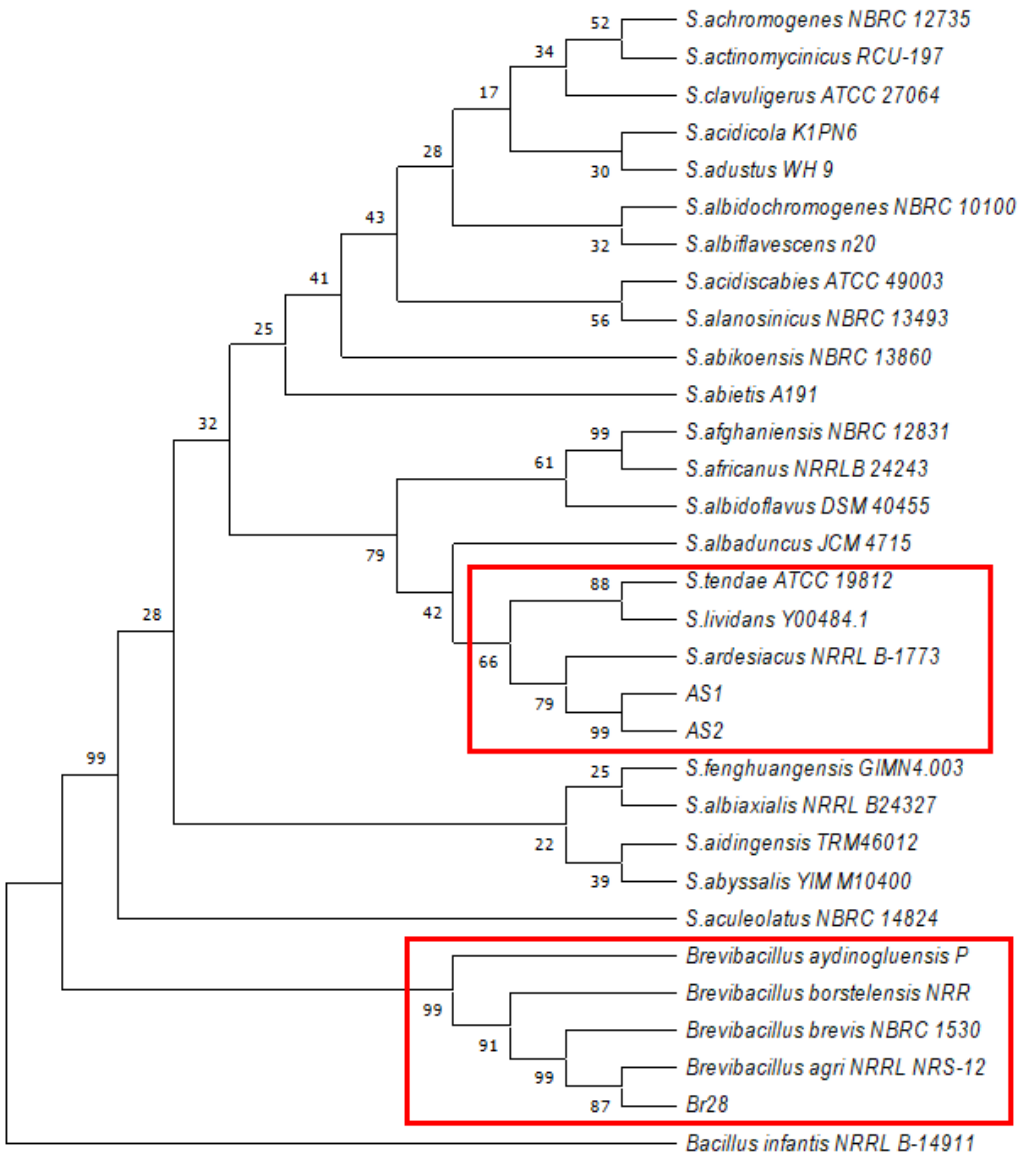

FIGURE 3 Molecular phylogenetic analysis by maximum likelihood method

results of the enzyme cut appropriately to vector length (5.4 kb). The length of the *merA* gene from *Pst*I to *Xho*I was approximately 1.1 kb. As shown in Figures 4a and 4b, the insertion of the *merA* gene into the vector pET-28c was correct. Figure 4c shows the results of cutting the pGEX-5x-1 plasmid using restriction enzymes. The vector length (4.9 kb) and the length of the *merA* gene from *Bam*HI to *Xho*I were equal to 1.4 kb. Cutting using *Bam*HI, *Pst*I, and *Xho*I produced 3 bands: vector 4.9 kb, 0.4 kb from *Bam*HI to *Pst*I, and 1.1 kb from *Pst*I to *Xho*I. This result showed that the *merA* gene was successfully cloned into an expression vector with the correct restriction site.

3.5. Discussion

This study described the mercury-resistant *Streptomyces* present in the *Cyperus rotundus* rhizosphere in the artisanal small-scale gold mining area in Lombok, Indonesia. Association between *Streptomyces* and nutgrass root system emergence the ability to face environmental stress due to mercury contamination. In situ mercury-bioremediation using indigenous microorganisms was reported for the first time in Indonesia (Winardi et al. 2020). However, identification of the microbes was not conducted. Identification of indigenous mercury-resistant bacteria is essential for effective bioremediation processes. Our study reported that *S. ardesiacus* AS1, *S. ardesiacus* AS2 and *B. agri* BR28 were mercury-resistant bacteria with potential for mercury transformation. These findings will enrich the information about mercury-resistant bacteria, especially from Indonesia whereas *Bacillus, Brevundimonas, Klebsiella, Pseudomonas*, and *Nitrococcus*, reportedly in the previous studies (Fatimawali et al. 2014; Zulaika and Sembiring 2015; Chasanah et al. 2018; Imron et al. 2019; Fatimawali et al. 2020).

The presence of indigenous bacteria is important for microorganism-assisted bioremediation in certain areas.

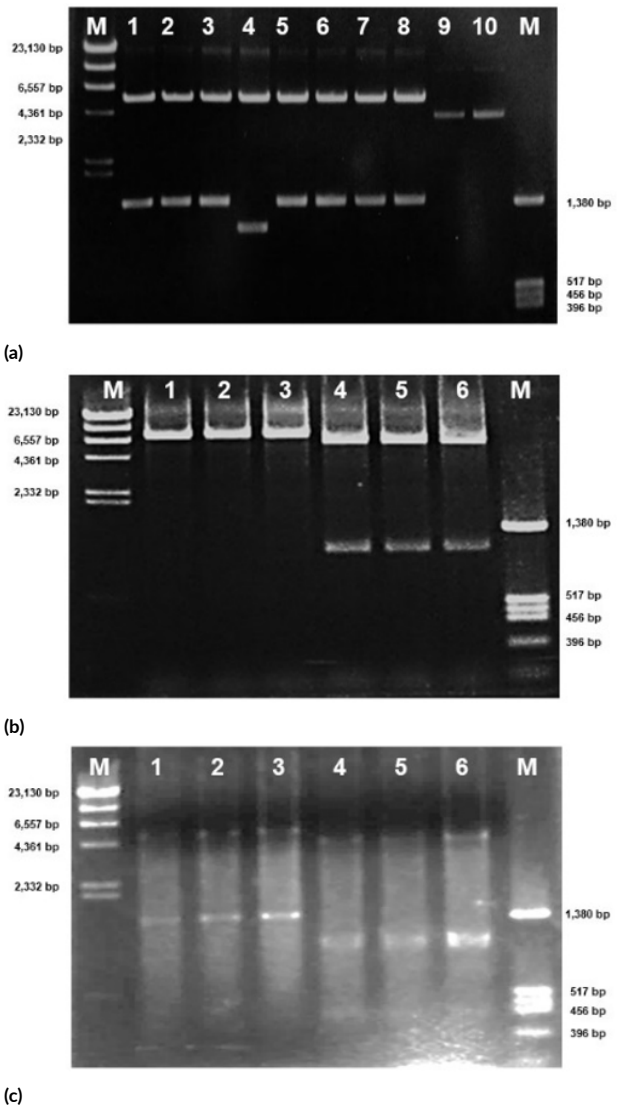

FIGURE 4 (a) pET-28c vector with *merA* restricted using *Bam*HI and *Xho*I, M: marker λ/*Hind*III, 1–8: transformant plasmid cut only with *Bam*HI and *Xho*I, 9–10: transformant plasmid not inserted with *merA*, M2: marker BSM 13. (b) pET-28c vector with *merA* restricted using *Pst*I and *Xho*I, M: marker λ/*Hind*III, 1–3: transformant plasmid cut only with *Pst*I, 4–6: transformant plasmid cut using *Pst*I and *Xho*I, M2: marker BSM 13. (c) Restriction sites on pGEX-5x-1 with *merA* inserted, M: marker λ/*Hind*III, 1–3: plasmids cut with *Bam*HI and *Xho*I, 4–6: plasmids cut using *Bam*HI, *Pst*I, and *Xho*I, M2: marker BSM 13.

Naturally, indigenous mercury-resistant bacteria will be more adaptive to climate and environmental conditions, resulting in more effective and efficient mercury biodegradation processes. Interestingly, the mercuric reductase is the key enzyme of mercury volatilization. This enzyme plays a role in reducing ionic mercury into a volatile form (Singh and Kumar 2020). This enzyme is encoded by *merA* gene, which is found in most mercury-resistant microorganisms.

Cloning of *merA* is essential to reveal how these genes work. Our study was successfully cloned using multiple primer sets and confirmed that the length of the *merA* gene is 1,425 bp and encoded as 474 aa. This finding was different from Fatimawali's *merA* study isolated from *Klebsiella pneumoniae*. The *merA* partial gene was successfully obtained 285 bp length, which encoded 94 aa due to using a single primer set (Fatimawali et al. 2014). Using multiple primer sets was recommended to obtain a full sequence of *merA* genes.

Recently, Zhang et al. (2021) developed an artificial mer operon which could detect, quantify and remove bioavailable heavy metal ions. In the future, gene cloning is predicted to be an important tool to develop biosensors, a suitable synthetic biological device to identify, detect and eliminate the bioavailable part of the environmental mercury or other heavy metal.

4. Conclusions

Based on the phylogenetic analysis of 16S rRNA gene sequences, the *Streptomyces* present in the rhizosphere of *Cyperus rotundus* in this study was classified into S. ardesiacus. Moreover, the full sequences of *merA* genes were successfully cloned in the cloning and expression vector with a confirmation length of 1,425 bp. Further cohort studies are needed to obtain recombinant protein expression in *E. coli*.

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Authors' contributions

WAP designed and performed the experiments and wrote the manuscript; HMR analyzed the data, AUK analyzed the data; and LS, MK, and YAP designed the experiments. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no conflict of interest in this research.

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