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≪ Research Paper ≫

Mercury Resistance and Removal Mechanisms of *Pseudomonas* sp. Isolated Mercury-contaminated Site in Taiwan

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ABSTRACT

A new strain of *Pseudomonas* sp. was isolated from mercury (Hg)-contaminated sites in Taiwan. This bacterium removed more than 80% of Hg present in the culture medium at 12 h incubation and was chosen for further analysis of the molecular mechanisms of Hg tolerance/removal abilities in this *Pseudomonas* sp. We used RNA-seq, one of the next-generation sequencing methods, to investigate the transcriptomic responses of the *Pseudomonas* sp. exposed to 60 mg/L of Hg^{2+}. We de novo assembled 4,963 contigs, of which 10,533 up-regulated genes and 5,451 down-regulated genes were found to be regulated by Hg. The 40 genes most altered in expression levels were associated with tolerance to Hg stress and metabolism. Functional analysis showed that some Hg-tolerant genes were related to the *mer* operon, sulfate uptake and assimilation, the enzymatic antioxidant system, the HSP gene family, chaperones, and metal transporters. The transcriptome were analyzed further with Gene Ontology (GO) and Cluster of Orthologous Groups (COGs) of proteins and showed diverse biological functions and metabolic pathways under Hg stress.

Key words : *Pseudomonas* sp., Transcriptome, Mercury, RNA-seq

1. Introduction

Heavy metals are a natural constituent of the environment. Mercury (Hg) is one of these toxic heavy metals and a widespread pollutant due to industrial contamination and its deposition in the ecosystem. The anthropogenic sources of Hg consist of some industries that produce chloralkali, paints, disinfectants, pulp, paper, catalysts, and fungicidal agents (Robinson and Tuovinen, 1984).

Prolonged exposure to such metal could cause deleterious effects on human life and aquatic biota (Singh et al., 2011). Although Hg is toxic to both eukaryotic and prokaryotic cells, some microorganisms have developed resistance mechanisms to the metal (Hobman and Crossman, 2015). Thus, bioremediation of contaminated soil with the use of microorganisms has emerged as the most safe and effective technology (Dixit et al., 2015). The role of some microorganisms in the biotransformation of heavy metals into non-toxic forms is well-documented. However, transcriptome studies focusing on genes involved in bacterial response to Hg have not been described using the high-throughput sequencing approach.

Using the next generation sequencing technology, a powerful tool to generate abundance of genes efficiently (Zhou et al., 2013), we could obtain a complete view of Hg-responsive genes expression in bacteria, and develop a natural strategy for the detoxification of Hg-contaminated sites based on the molecular basis of plasmids or transposan borne mercurial resistance.

2. Materials and Methods

2.1. Isolation of Hg-resistant bacteria

Soil samples were collected from a Hg-contaminated site in Southern Taiwan. Hg-resistant bacteria were isolated using the primary enriched method. Soil (3 g) samples were added to 8 mL of Luria-Bertan broth (LB) medium containing $HgCl_2$ (20 ppm) and incubated for 16 h at 30ºC. An aliquot of 1 mL of the enriched culture medium was incubated in LB containing $HgCl_2$ (40 ppm), as described above. These

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same inoculation and incubation procedures were repeated until the addition of $HgCl_2$ reached 60 ppm in the LB medium. Enrichment culture was serial dilution 10^{-1} to 10^{-8} and 100 μL of the enrichment culture was then spread on an LB agar medium supplemented with 80 ppm of Hg, followed by incubation at 30ºC for 16 h. A single colony was selected for 16S rRNA sequencing. The identification of bacterial isolates from the enrichment culture was determined by 16S rRNA gene sequence analysis as well as their basic physiological and biochemical characteristics. The 16S rRNA genes of the selected isolates were amplified, sequenced, and compared with the 16S rRNA gene sequences available in the NCBI (National Center for Biotechnology Information) public database as described in a prior study (Chien et al., 2014).

2.2. Removal of Hg by *Pseudomonas* sp.

This bacterium was grown overnight in LB containing $HgCl_2$ (20 ppm) at 30ºC for 18 h. Subsequently, this bacterium was inoculated into LB medium containing 60 ppm of HgCl2 at initial optical density (OD) 0.05 for the indicated times. The growth of this bacterium was determined by reading the OD600, and the Hg removal capacity was determined from the percent of Hg remaining. The concentrations of Hg remaining in the medium with and without the inoculated bacterium were determined according to the Taiwan EPA Method 245.1. All of the glassware needed to be washed with 30% HNO_3 and rinsed several times in ultrapure water prior to use. An aliquot of supernatant (1 mL) was added to the tubes containing a 50 μL H_2SO_4 and 25 μL HNO3. Subsequently, $KMnO_4$ was added to the tubes and was autoclaved for 2 hours at 95ºC. After adding 12% hydroxylamine hydrochloride, the remaining $KMnO_4$ was reduced. The Hg was quantified in a cold vapor atomic absorption spectrometer (PerkinElmer, USA).

2.3. RNA isolation, integrity examination, and RNA-seq library preparation

Total RNA was extracted from the *Pseudomonas* sp. treated with and without $HgCl_2$ by using Total RNA Mini Purification Kit (P&C Biotech, TW) according to the manufacturer's instructions. RNA integrity was confirmed by the 2100 Bioanalyzer (Agilent). The paired-end library was constructed with the Ovation® Complete Prokaryotic RNA-Seq DR Multiplex System (NuGen, CA, United States) (Hsu et al., 2016). All procedures were performed according to the respective manufacturers' instructions. The libraries concentration and quality were assessed by the 2100 Bioanalyzer using a DNA 1000 lab chip (Agilent, Palo Alto, CA). The libraries were sequenced using a paired-end read protocol with 2 × 100 bp of data collected per run on the Illumina HiSeq2000 sequencing platform. Data analysis and base calling were performed by the software in the Illumina instrument. Sequencing was performed using the HiSeq2000 platform (Illumina, USA) according to the manufacturer's instructions. The analysis of sequencing data was performed with CLC Genomics Workbench v 8.0 (CLC bio, Denmark), and the reads of the gene sequences with Ns or an overall mean Q score of < 20 were discarded. After filtering the low-quality reads, the raw reads were cleaned up by removing adapter sequences. De novo assembly of the short reads was performed using CLC Genomics Workbench v 8.0. At least 80% of the length and the similarity of reads should be mapped to a reference transcript to construct contigs. The contigs were joined into scaffolds using the paired-end reads. The paired-end reads were also used to fill the gaps in scaffolds where the unigenes had the least Ns and could not be extended on both ends. Then the FPKM value for each transcript was measured. If the value of FPKM in the presence of the inoculated bacteria in the medium containing $HgCl_2$ (or not) was zero, we used 0.01 instead of 0 to calculate the fold change (Hsu et al., 2016). The false discovery rate (FDR) method was used to determine the threshold of the p value. In this study, we used FDR ≤ 0.05 and the absolute value of the ratio ≥ 2 as the threshold to judge the significance of the differentiated gene expression.

2.4. Gene Ontology (GO) functional enrichment analysis for differentially expressed genes (DEGs)

Functional analysis was performed for differentially expressed genes from each group using Gene Ontology terms. We firstly mapped all DEGs to GO terms in the database (http://www.geneontology.org/) by virtue of calculating gene numbers for every term, which was followed by an ultrageometric test to find significantly enriched GO terms

in DEGs when comparing the transcriptome background in two libraries. The calculated p value was subjected to a Benjamini-Hochberg correction. A corrected p value of 0.05 was used as the threshold for judging the significantly enriched GO terms in DEGs.

3. Results

3.1. Identification of the microorganisms

The analysis of the 16S rRNA gene sequence identified our isolated bacterium from the Hg-contaminated site as a *Pseudomonas* sp. Several studies have shown that bacteria of the genus *Pseudomonas* are highly resistant to the different forms of Hg (Hobman and Crossman, 2015; Ndu et al., 2016) and have been reported in great abundance in waste high in Hg concentrations.

3.2. Capacity for Hg removal

The bacterium was capable of removing 50 ppm Hg from the culture medium after 24 h of incubation (Fig. 1), although we found that a decrease of Hg due to its vaporization into air in the control group. In addition, this bacterium did not grow at all when the concentration of Hg in the LB medium was increased to 100 ppm (data not shown), indicating that a higher Hg concentration was toxic to the bacterial growth.

3.3. Transcriptome assembly and sequence annotation

Although some studies had already reported that *Pseudomonas* spp. could remove Hg, the detailed mechanisms of Hg removal by *Pseudomonas* spp. remained unknown. Thus, the isolation of RNA collected from the sampling times of 0 h and 24 h was performed via RNA-seq to obtain the global transcriptome response in the *Pseudomonas* sp. for Hg removal. RNA-seq, a high throughput sequencing method, is a rapid and revolutionary technique relative to traditional methods (Wang et al., 2009). The global transcriptome information on the *Pseudomonas* sp. treated with Hg was uncovered here for the first time and thus could provide the molecular basis for the toxicity of Hg to *Pseudomonas* sp.

After the *Pseudomonas* sp. was treated with Hg, we extracted total RNA from the treated or nontreated bacteria

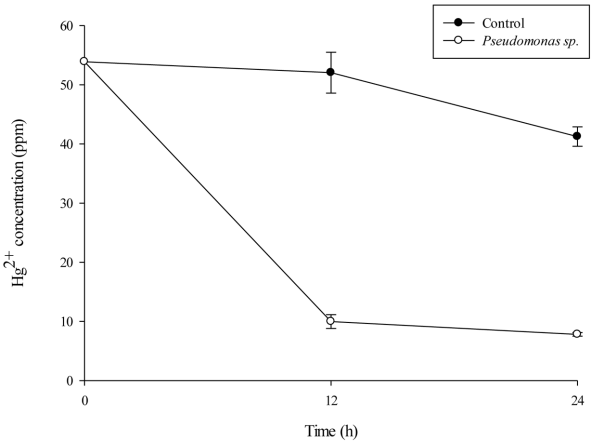

Fig. 1. Dynamic removal of Hg and growth of *Pseudomonas* sp. in the presence of Hg (60 ppm) and absence of Hg. Points represent the average of 3 replicates, and the error bars represent the standard deviation.

to analyze the transcriptional regulation using RNA-seq. Firstly, we observed that 25,441,327 and 26,475,557 clean reads were generated from the 2 groups, respectively, resulting in a total number of 45,998,434 bp paired-end reads (data not shown). The assembly yielded a total of 3,826 contigs, with an average length of 5,170 bp. The prediction of the open reading frame (ORF) within the contigs was carried out by Glimmer (Tao et al., 2015). BLASTX searches using the Blast2Go program against the NCBI nonredundant protein database revealed sequence homologies when the *E* value of was set as $\leq 10^{-3}$. Between 76.44% and 76.19% of the reads of each sample could be mapped (Table 1).

The sequences revealing significant BLASTX hits were further annotated according to GO terms. The total mapped sequences using a cutoff of 2-fold change in gene expression (corrected p value < 0.05) could be assigned to GO terms representing a broad range of important biological processes, cellular components, and molecular functions. A total of 10,533 up-regulated genes and 5,451 down-regulated genes were observed. These up-regulated genes could be assigned to GO terms representing a broad range of important biological processes and molecular functions (Fig. 2). The wide spectrum of BLASTX hits and GO annotations of the ORF in the assembled contigs indicated that a large proportion of genes were expressed in the *Pseudomonas* sp. under Hg stress.

Table 1. Number of total reads and contigs obtained per sample as well as number and percentage of reads per sample mapping on the assembled transcriptome

	60 ppm Hg^{2+}	Control
Total base pair (bp)	2,695,906,140	2,592,543,346
Total number of Reads	26,692,140	25,668,746
GC percentage	61.6%	62.50%
Total number of contigs	4,963	
Mean length of contigs (bp)	3,983	
Total number of scaffolds	3,826	
Mean length of scaffolds (bp)	5,170	

Sample	Read type	No.reads	Mapped reads	%mapped
-Hg	Pair end	25,668,746	19,622,032	76.44
Hg	Pair end	26,692,140	20,337,622	76.19
Total	Pair end	52,360,886	39,959,654	76.31

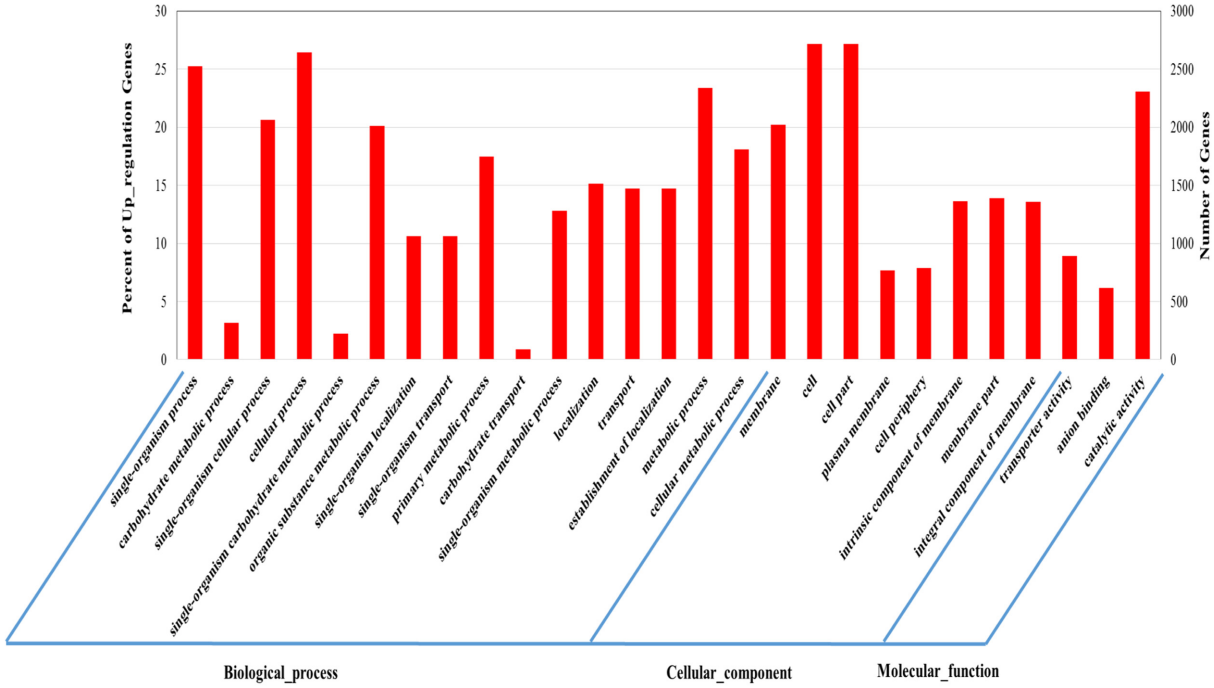

Fig. 2. Gene Ontology classification of ORF in the assembled contigs. The results are summarized in three categories: "biological process," "molecular function," and "cellular component".

3.4. Functional categorization of differentially expressed genes by Cluster of Orthologous Groups (COGs) of proteins

We used the functional catalogues of COGs (Qin et al., 2011) to identify which biological functions were influenced by Hg. All sequences of the transcriptome that could be identified by BLASTX were classified. The genes represented in the differentially expressed subsets ($p < 0.05$, corrected $p < 0.05$) were classified. Concerning genes that were up-regulated in response to Hg, we found that these genes were classified into many categories. To demonstrate which genes could be strongly regulated by Hg stress, we used the 40 genes that were most differentially expressed in the 2 libraries (Table 2). Among these, 6 genes could be grouped together. These genes are related to cell wall/membrane/envelope biogenesis, including UDP-galactopyranose mutase

Table 2. The 40 Most differentially expressed genes between Hg-Treated (+Hg) and Hg-Free (-Hg) Libraries of *Pseudomonas* sp. Based on the expressed read frequency. Log_2 (Hg/-Hg) > 0 Indicated Genes Up-Regulation and Log_2 (Hg/-Hg) < 0 Indicated Gene Down-Regulation

GI	Log_2 (Hg/-Hg)	Annotation	COG class
gi|16128232	12.59	CP4-6 prophage; predicted DNA repair protein	[L] Replication, recombination and repair
gi|495380142	12.44	UDP-galactopyranose mutase	[M] Cell wall/membrane/envelope biogenesis
gi|525858865	12.42	DNA-binding protein	
gi|545153713	12.33	ATPase	
gi|490301073	12.31	Type-1 restriction enzyme EcoKI specificity protein	[V] Defense mechanisms
gi|493838846	12.29	DNA polymerase subunit V UmuC	[L] Replication, recombination and repair
gi|152973424	12.28	ATP-dependent helicase	[R] General function prediction only
gi|260600056	12.28	Probable sensor protein pcoS	[T] Signal transduction mechanisms
gi|490305698	12.26	Phage exclusion protein	
gi|157412124	12.23	Copper/silver efflux system outer membrane protein CusC	[M] Cell wall/membrane/envelope biogenesis
gi|545153711	12.23	Mechanosensitive ion channel protein MscS	[M] Cell wall/membrane/envelope biogenesis
gi|386706749	12.22	Putative membrane fusion protein SilB	[M] Cell wall/membrane/envelope biogenesis
gi|486200968	12.22	Prophage integrase	[L] Replication, recombination and repair
gi|493366908	12.22	Integrase	[L] Replication, recombination and repair
gi|383189645	12.19	Sugar ABC transporter periplasmic protein	[G] Carbohydrate transport and metabolism
gi|486159953	12.19	Alkaline phosphatase	
gi|485693110	12.19	ATPase	
gi|545151495	12.18	Vitamin B12/cobalamin outer membrane transporter	[H] Coenzyme transport and metabolism
gi|545152877	12.18	F0F1 ATP synthase subunit gamma	[C] Energy production and conversion
gi|16767735	12.17	ATP-dependent Lon protease	[O] Posttranslational modification, protein turnover, chaperones
gi|485755705	12.16	Copper-translocating P-type ATPase	[P] Inorganic ion transport and metabolism
gi|386706748	12.16	SilA	[P] Inorganic ion transport and metabolism
gi|545153419	12.16	MFS transporter	[G] Carbohydrate transport and metabolism
gi|545153766	12.16	Diguanylate phosphodiesterase	[T] Signal transduction mechanisms
gi|545152748	12.15	DeoR family transcriptional regulator	[K] Transcription
gi|545153387	12.15	Pantothenate kinase	[H] Coenzyme transport and metabolism
gi|525858873	12.12	DNA topoisomerase III	[L] Replication, recombination and repair
gi|545153543	12.11	Tar	[N] Cell motility
gi|317047017	12.11	Lytic transglycosylase	[M] Cell wall/membrane/envelope biogenesis
gi|525860234	12.08	LysR family transcriptional regulator	[K] Transcription
gi|545151743	12.08	Glycosyltransferase	[M] Cell wall/membrane/envelope biogenesis
gi|545154228	12.08	Membrane protein	[T] Signal transduction mechanisms
gi|545152724	12.06	Homoserine O-succinyltransferase	[E] Amino acid transport and metabolism
gi|545151496	12.06	tRNA (uracil-5-)-methyltransferase	[J] Translation, ribosomal structure and biogenesis
gi|545151254	12.05	Polyphosphate kinase	[P] Inorganic ion transport and metabolism
gi|545151990	12.05	LysR family transcripitonal regulator	[K] Transcription
gi|545154217	12.05	Acyltransferase	[I] Lipid transport and metabolism
gi|545152564	12.04	Rhizopine-binding protein	[G] Carbohydrate transport and metabolism
gi|545152912	12.04	Preprotein translocase subunit SecY	[U] Intracellular trafficking, secretion, and vesicular transport
gi|545153831	12.04	Type VI secretion protein VasK	[S] Function unknown

(gi|495380142), copper/silver efflux system outer membrane protein CusC (gi|157412124), mechanosensitive ion channel protein MscS (gi|545153711), putative membrane fusion protein SilB (gi|386706749), lytic transglycosylase (gi|317047017),

Table 3. Metal-tolerant genes differentially expressed in the control (-Hg) and Hg-Treated (+Hg) *Pseudomonas* sp.

Gene ID	Log_2 (Hg/-Hg)	Annotation
Mer operon		
gi|32455838	10.01	MerA protein
gi|49188503	11.04	MerR
gi|32455840	9.36	MerP protein
gi|146283749	9.97	Mercuric transport protein
Sulfate uptake and assimilation		
gi|545153612	11.87	Glutathione ABC transporter permease GsiD
gi|545152569	11.72	Glutathione S-transferase
gi|545153607	11.67	Glutathione ABC transporter ATP-binding protein
gi|545151303	11.50	Glutathione synthetase
gi|545150913	11.33	Glutathione peroxidase
Enzymatic antioxidant system		
gi|545150879	10.99	Superoxide dismutase
gi|545152528	11.85	Catalase
gi|544902114	11.80	Thioredoxin
gi|545153050	11.77	Thioredoxin reductase
HSP gene family		
gi|446272580	11.81	Heat shock protein Hsp20
gi|545151896	11.77	Heat shock protein 90
gi|545154727	11.55	Heat shock protein HtpX
gi|545152990	10.66	Heat shock protein HspQ
gi|545151183	11.39	Heat shock protein GrpE
Chaperone		
gi|545153355	12.02	Molecular chaperone DnaK
gi|545151993	12.01	Protein disaggregation chaperone
gi|545152446	11.85	Molecular chaperone GroEL
gi|545152447	11.60	Co-chaperonin GroES
gi|545153356	11.48	Molecular chaperone DnaJ
Metal transporter		
gi|493838830	11.98	Heavy metal translocating P-type ATPase
gi|545151426	11.74	Metal ABC transporter substrate-binding protein
gi|545154479	11.71	Metal ABC transporter permease
gi|167031051	10.23	Heavy metal transport/detoxification protein
gi|512581402	10.37	Cation transporter
gi|148548493	10.44	CzcA family heavy metal efflux protein
gi|490714248	10.09	Heavy metal translocating P-type ATPase

and glycosyltransferase (gi|545151743). Changes in the expression of the above genes could be interpreted to suggest heavy metals' ability to modify cell walls. There were 3 genes encoding the sugar transporter (namely, sugar ABC transporter periplasmic protein, MFS transporter, rhizopine-binding protein). Similarly, the up-regulation of sugar-catabolizing enzymes was observed in Elodea nuttallii (Regier et al., 2013) treated with Hg. The other genes encoded proteins for diverse biological function. It is worth noting that there was a gene coding for probable sensor protein pcoS, which has been shown to act as one of the regulator proteins for the synthesis of plasmid copper-resistant systems in *Escherichia coli* (Munson et al., 2000). Briefly, the up-regulation of these top-40 genes is involved in bacterial responses to Hg stress.

4. Discussion

Illumina sequencing has been proven to be suitable for de novo assembly (Kisan and Lettieri, 2013) and has also been applied successfully to assemble the transcriptomes of *Eucalyptus* (Vining et al., 2015), sweet potatoes (Xie et al., 2012), chickpeas (Pradhan et al., 2014), and *Taxus* (Sun et al., 2013). De novo assembly was able to generate high-quality sequence information when it was applied to the nonmodel organisms with no or scarce sequence information analyzed. *Elodea nuttallii* transcriptome in response to Hg pollution using Illumina sequencing (Regier et al., 2013), indicating that Illumina sequencing was a sensitive tool for rapid ecotoxicological testing.

Microorganisms that are used in bioremediation processes should have acquired Hg-resistance mechanisms (Cursino, et al., 2000), including uptake and reflux contaminating metals, complexion, precipitation, and their release. In this study, we identified many Hg-resistant genes, which could enable us to delineate the mechanisms of Hg resistant in *Pseudomona*s sp we isolated. A group of metal transporters we identified can be classified into different families based on their location in cells and the metals they carry as was reported by Singh et al (2015). Three genes coding for heavy metal transporters (2 heavy metal-translocating P-type ATPases and 1 heavy metal transport/detoxification protein) were identified (Table 3). The expression of heavy metal-translocating P-type ATPases was also induced in Medicago truncatula treated with Hg (Singh et al., 2015), suggesting that these transporters are involved in counteracting Hg stress. Some specific sulfate-containing compounds such as glutathione form complexes with heavy metals for the detoxification of metal toxicity (Jozefczak et al., 2012). An excess of Cd/Zn could activate sulfate via a group of sulfate transporters (Sun et al., 2007) and sulfur assimilation pathways, leading to the synthesis of glutathione which could protect the bacteria from oxidative stress. Thus, the response of sulfate uptake and assimilation is considered a crucial process for the survival of bacteria under Hg stress. Additionally, Hg increased the expression of superoxide, catalase, thioredoxin, and thioredoxin reductase, which suggests that these genes could provide tolerance of bacteria to Hg-induced oxidative stress. As heat shock proteins have been reported to be induced by toxic metals (Cassier-Chauva and Chauvat, 2015), five genes coding for heat shock proteins and five genes coding for chaperons identified in this study were suggested to be able to protect bacterial cells from Hg stress due that they are known to assist in protein folding and reintegration into the membrane. The *mer* operon is one of the most widely distributed in the Hg detoxification genetic system (Mathema et al., 2011). Various genes are involved in the *mer* operon, including *mer*R/*mer*D for detection, *mer*P/*mer*T/*mer*C for transportation or mobilization, and *mer*B/*mer*A for enzymatic detoxification of inorganic and organic Hg compounds in bacteria. In this study, Hg induced the expression of some genes (*mer*A, *mer* R, *mer*P, and *mer*T) in the *mer* operon, as expected. Most of the *mer* operons contain a regulatory gene, *mer* R, which is transcribed separately and divergently from the functional *mer* genes, which could bind with the promoter-operator region to positively and negatively regulate the *mer* operon (Dash and Das, 2012). The expression of *mer*A concentration is regulated by the Hg concentration. The role of *merA* gene coding for mercuric ion reductase can transform Hg (2+) into non harmless Hg (0). Most of *mer* operons contain *mer* T and *mer* P which help in the transport of mercuric ions into the cytoplasm.

In conclusion, we have presented an extensive survey of Hg-responsive genes, showing differential expression in bacteria. These genes were involved in many biological responses. Our results not only demonstrate the transcriptional complexity in bacteria with Hg but also provide detailed information for identifying genes responsible for the regulation of Hg tolerance/removal abilities. Furthermore, the high-throughput sequencing platform described here serves as powerful research for improving the ability of Hg tolerance/removal abilities in bacteria based on the modification of these possibly potential genes detected by RNA-seq.

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strued as the opinions of the Taiwan EPA. The mention of trade names, vendor names, or commercial products does not constitute endorsement or recommendation by the Taiwan EPA.

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