The Construction of an Engineered Bacterial Strain and Its Application in Accumulating Mercury from Wastewater

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Abstract: To remove organic and inorganic mercury from wastewater, an engineered bacterial strain, BL21-7, was constructed that contained the artificial operon *P16S-g10-merT-merP-merB1-merB2-ppk-rpsT*. For BL21-7, the minimum inhibitory concentrations of mercuric chloride, methylmercury chloride and phenylmercury chloride in Luria-Bertani (LB) medium were 100 μmol/L, 60 μmol/L and 80 μmol/L, respectively. After being cultured in three media (liquid LB containing 80 μmol/L mercuric chloride, 40 μmol/L methylmercury chloride or 60 μmol/L phenylmercury chloride) for 72 h, the engineered bacteria accumulated up to 70.5 ± 1.5 μmol/L, 33.5 ± 3.2 μmol/L and 45.3 ± 3.7 μmol/L of mercury, respectively. In the presence of 10 μmol/L Cd^{2+}, 10 μmol/L Pb^{2+} or 10 μmol/L Cu^{2+}, the accumulation of mercurial derivatives by BL21-7 was not affected. BL21-7 could accumulate mercury well in media with pH values ranging from 5 to 8 and it could work well at temperatures from 25 °C to 37 °C. After BL21-7 was added to wastewater and cultured for 24 h, approximately 43.7% of the Hg in the wastewater was removed.

Keywords: engineering bacteria; mercury; accumulation

1. Introduction

Mercury (Hg) pollution threatens public health. In nature, Hg exists in three forms: elemental (Hg(0)), inorganic (Hg^{2+} or Hg^+) and organic forms (R-Hg^+ or R-Hg-X, where "R" is methyl or phenyl and "X" is acetate) [1]. Elemental Hg is volatile. Inorganic Hg commonly exists as mercuric sulfide and is used as a coloring agent in tattoos and paints. All the forms are hazardous but the extent of toxicity varies among different forms of Hg. Organic Hg is the most toxic and elemental is the least toxic. Elemental Hg can potentially become an inhalation hazard. Organic Hg can impair neurological development in fetuses and young children and can damage the central nervous system of adults [2,3]. Exposure to inorganic Hg may damage the gastrointestinal tract, the nervous system and the kidneys [4]. The Hg in agricultural soil can enter the human body through crops and Hg mainly comes from irrigated-polluted water released from factories [5]. The best resolution for this problem is removing Hg from polluted water before it is discharged from factories.

Engineered bacterial strains can be used to remove Hg from polluted water. After *E. coli* JM109 cells, transformed with the mouse metallothionein gene *mt-1* were cultured in LB medium containing 120 μmol/L mercuric chloride ($HgCl_2$) for 72 and 120 h, the bacteria accumulated 51.6 μmol/L Hg and 1002 μmol/L Hg, respectively [6]. However, wild-type untransformed *E. coli* JM109 cells

(the control) could not grow at all in LB medium containing 120 μmol/L $HgCl_2$ [6]. However, it is unclear whether the strain accumulated organic Hg, a main pollution component in wastewater [6]. Bacteria harboring pMRA17 containing the *Pseudomonas* K-62 genes *merR*, *merT*, *merA* and *merB1* had a higher methylmercury tolerance than the control [7]. However, their Hg accumulation was achieved by volatilizing Hg(0) into the air, which causes public anxiety. The same problem exists in the *Cupriavidus metallidurans* strain MSR33, which harbors two large plasmids (pMOL28 and pMOL30) carrying genetic determinants for heavy metal resistance [8]. Although bacteria harboring pMRB01, which contains *Pseudomonas* K-62 genes *merR*, *merB2* and *merD*, had a higher tolerance to $HgCl_2$ than the control, they were hypersensitive to phenylmercury [7]. *E. coli* harboring pMKB18, which contains *Pseudomonas* K-62 genes *merR*, *merT*, *merP* and *merB1* as well as the *Klebsiella aerogenes* polyphosphate kinase gene *ppk* could remove 55% of the Hg^{2+} and 31% of the $C_6H_5Hg^+$ when it was cultured in medium containing 80 μmol/L Hg^{2+} or 80 μmol/L $C_6H_5Hg^+$ for 20 h [9] but whether it can accumulate CH_3Hg^+ has not been reported. CM111 and HG3, two bacteria isolated from Hg-polluted rivers or sand, also had high minimum inhibitory concentration (MIC) values of Hg^{2+} [10]. Although the tolerance of CM111 and HG3 to Hg^{2+} was not due to Hg(0) volatilization [10], the detailed mechanisms of these two strains' tolerance to Hg^{2+} are unclear. Whether these strains can remove organic Hg from a medium is also unknown.

The aim of this study was to construct an engineered bacterial strain for accumulating Hg from liquid medium. This bacterial strain should meet the following criteria: (1) it can accumulate both mercuric ions and organic mercury; (2) it has a clear genetic background; (3) its mechanisms for mercury accumulation are definite; and (4) it can keep mercury accumulated in cells. To achieve this aim, an artificial operon, *P16S-g10-merT-merP-merB1-merB2-ppk-rpsT* and a bacterial strain, BL21-7, were constructed. The role of the genes in the artificial operon and the mechanism by which the bacteria accumulated mercury were also studied.

2. Materials and Methods

2.1. Strains and Chemicals

A genomic DNA extraction kit and 2× Pfu MasterMix were purchased from ComWin Biotech (Beijing, China). An RNeasy Mini kit was obtained from Qiagen (Venlo, The Netherlands) and an AccuScript cDNA kit was acquired from Stratagene. An In-Fusion HD Cloning Kit, T4 DNA ligase, $HgCl_2$, methylmercury chloride (MMC), phenylmercury chloride (PMC) and other chemicals were purchased from TAKARA (Dalian, China). *Enterobacter aerogenes*, *E. coli* DH10B, *E. coli* BL21 (DE3), pET28a plasmid and Tobacco NC89 seeds were stocked in our laboratory.

2.2. Constructing the Engineered Bacterial Strains

To construct the artificial operon *P16S-g10-merT-merP-merB1-merB2-ppk-rpsT*, a series of primers were designed and synthesized (Table 1). Based on the sequences of plasmids pMR26 (GenBank accession number: D83080.2) and pMR28 (GenBank accession number: AB013925.1), the artificial operon *merT-merP-merB1-merB2* was designed and synthesized by TAKARA (Dalian, China). The synthesized DNA fragment was inserted into the region between the *Nde* I and *Not* I restriction sites of plasmid pET28a using an In-Fusion HD Cloning Kit (Clontech, code number: 639633). The resulting plasmid was named p1.

The *ppk* gene was amplified from *Enterobacter aerogenes* genomic DNA using 2× Pfu MasterMix (ComWin Biotech, code number: CW0686A). The primers used were PPPKF and PPPKR. The amplified fragment was inserted into the area between the *Not* I and *Xho* I restriction sites of plasmid p1. The resulting plasmid was named p2 (Table 1). According to published DNA sequences (GenBank accession number: Z00044.2, reference) [11], P1F and P1R were synthesized and 1 mmol/L P1F and 1 mmol/L P1R were mixed with 25 μL of 2× Pfu MasterMix. The volume of reaction was 500 μL. The mixture was annealed in a PCR amplifier. The annealed product, the strong constitutive

promoter *P16S* [12–14] and the translation enhancer *g10* [11], was inserted into plasmid pET28a. The resulting plasmid was named p3 (Table 1). Primers P2F and P2R were synthesized and DNA fragment *merT-merP-merB1-merB2-ppk* was amplified using p2 as a template. The amplified fragment was inserted into the region between the *Bal* I and *Eco*R I sites of p3. The resulting plasmid was named p4 (Table 1).

Table 1. The primers used in this study.

Primer	Sequence	Plasmid
PPPKF	5′-ATAAGAAT<u>GCGGCCGC</u>ATTTACCACGTCCTGTGATT-3′; *Not* I restriction site is underlined.	p2
PPPKR	5′-CCGATCCGC<u>TCGAG</u>GGGTTAATCGGGTTGCTCGAG-3′; *Xho* I restriction site is underlined.	p2
P1F	5′-CGA<u>CATATG</u>GCTCCCCCGCCGTCGTTCAATGAGAATGGATAAGAGGCTCGTGGG ATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATA CGAAGCGCTTGGATACAGTTGTAGGGAGGGATTTATCTTTTAACTTTAAGAAGGAG T<u>GGCCA</u>AGCGCTATTCGATCGA<u>CATATG</u>GGAC-3′ *Nde* I and *Bal* I restriction sites are underlined.	p3
P1R	5′-GTCCCATATGTCGATCGAATAGCGCT<u>TGGCCA</u>CTCCTTCTTAAAGTTAAAAGATAA ATCCCTCCCTACAACTGTATCCAAGCGCTTCGTATTCGCCCGGAGTTCGCTCCCAGAA ATATAGCCATCCCTGCCCCCTCACGTCAATCCCACGAGCCTCTTATCCATTCTCATTGA ACGACGGCGGGGGAGC<u>CATATG</u>TCG-3′ *Nde* I and *Bal* I restriction sites are underlined.	p3
P2F	5′-CGG<u>TGGCCA</u>GTATGTCTGAACCACAAAAACG-3′; *Bal* I restriction site is underlined.	p4
P2R	5′-CGC<u>GAATTC</u>TTATCATTTATCGGCATAGGT-3′; *Eco*R I restriction site is underlined.	p4
P3F	5′-CCG<u>GAATTC</u>CGTGTTCTATAACGACAAAA-3′; *Eco*R I restriction site is underlined.	p5
P3R	5′-CCG<u>GAATTC</u>AAGAAAATATCGAAGAAAAAT-3′; *Eco*R I restriction site is underlined.	p5

Primers P3F and P3R were synthesized according to a previously published sequence (GenBank accession number: Z00044.2). Using P3F and P3R as primers, *rpsT*, a strong terminator that can enhance transcript stability by forming a secondary structure at the 3′ end of the mRNA [15], was amplified from tobacco chloroplast DNA. The amplified *rpsT* was inserted into the *Eco*R I site of p4. The resulting plasmid, in which the transcription direction of *rpsT* was consistent with that of *merT-merP-merB1-merB2-ppk*, was named p5 (Table 1). The *E. coli* BL21 strain harboring p5 was named BL21-7. The sequence of the artificial operon *P16S-g10-merT-merP-merB1-merB2-ppk-rpsT* is shown in Supplementary Materials S1. To characterize the roles of the genes in the artificial operon *P16S-g10-merT-merP-merB1-merB2-ppk-rpsT* and the transcription of the operon, a series of artificial operons were constructed (Table 2). The detailed procedures can be found in Supplementary Materials S2.

Table 2. The constructs of the artificial operons and the corresponding bacterial strains.

Bacterial Strains	Plasmids Harboring Artificial Operons
BL21-0	pET28a
BL21-1	pET28a (*g10-ppk-rpsT*)
BL21-2	pET28a (*P16S-g10-ppk-rpsT*)
BL21-T	pET28a (*P16S-g10-merT-rpsT*)
BL21-P	pET28a (*P16S-g10-merP-rpsT*)
BL21-B1	pET28a (*P16S-g10-merB1-rpsT*)
BL21-B2	pET28a (*P16S-g10-merB2-rpsT*)
BL21-TP	pET28a (*P16S-g10-merT-merP-rpsT*)
BL21-B1B2	pET28a (*P16S-g10-merB1-merB2-rpsT*)
BL21-TPK	pET28a (*P16S-g10-merT-merP-ppk-rpsT*)
BL21-B1B2K	pET28a (*P16S-g10-merB1-merB2-ppk-rpsT*)
BL21-TPB1B2	pET28a (*P16S-g10-merT-merP-merB1-merB2-rpsT*)
BL21-7	pET28a (*P16S-g10-merT-merP-merB1-merB2-ppk-rpsT*)

2.3. Real-Time Reverse-Transcription PCR

The *E. coli* BL21 strain transformed with empty plasmid pET28a was named BL21-0. BL21-0, BL21-1, BL21-2 and BL21-7 were cultured in Luria-Bertani (LB) liquid medium for 16 h at 37 °C. Bacterial total RNA was extracted from 1 mL of these cultures using a Column Bacterial RNA Extraction kit (ABiGen, Beijing, China). Reverse transcription was performed using a PrimeScript RT Reagent Kit (TAKARA, Dalian, China). This kit contains a DNA eraser that can remove genomic DNA from RNA samples without requiring purification. The cDNA was analyzed using StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) and a two-step amplification program was used for the analysis. SYBR Premix Ex Taq II (TAKARA, Dalian, China) was used for real-time PCR. To prevent an effect of the *ppk* gene of the bacterium itself, *ppk-rpsT* was used as a candidate for detection. The sequences of the primers for *ppk-rpsT* overlapped both *ppk* and *rpsT*. The forward primer PPRF (5′-AACTTTATCGAAAACCCGTACCGTC-3′) was within the region of *ppk*. The reverse primer PPRR (5′-ATTTATTTAATCCATAATGGATTCA-3′) was within the region of *rpsT*. The primers were diluted in a series from 1×10^7 copies/μL to 1×10^2 copies/μL to obtain a quantitative standard curve. Experiments were repeated four times and the average values were used.

2.4. Measuring Bacterial Hg Tolerance

A series of tubes were filled with 5 mL of fresh LB liquid medium. A stock solution of $HgCl_2$ (0.1 mol/L) was initially prepared and a series of $HgCl_2$ stock solutions were added to the medium to final concentrations of 0, 5, 10, 20 and 40 μmol/L. Another 5 mL of fresh LB liquid medium was placed in a different series of tubes. An MMC stock solution (1 mmol/L) and a PMC stock solution (1 mmol/L) were also prepared. A series of MMC stock solutions were added to the medium to final concentrations of 0, 50, 100, 200 and 400 nmol/L. A series of PMC stock solutions were also added to the medium to final concentrations of 0, 50, 100, 200 and 400 nmol/L. After BL21-0 and BL21-7 were cultured in LB at 37 °C for 16 h, the cultures were placed in LB liquid medium containing organic Hg or $HgCl_2$ at an OD_{600} = 0.01. The tubes were then placed in a shaker and cultured at 37 °C for 5 h. IPTG (Isopropyl β-D-Thiogalactoside) was then added to create a final concentration of 0.8 mmol/L. The cultures were then shaken at 37 °C for an additional 16–20 h. The light absorption values were measured at 600 nm. Each sample was measured with three replicates and the average value was used.

2.5. Measuring Minimum Inhibitory Concentration (MIC) Values

The bacterial MICs of the mercuric varieties and other metals were measured in two ways. The first was using the method described in "measuring bacterial Hg tolerance" but the final concentrations of the mercuric varieties in the medium were 3, 5, 10, 20, 40, 60, 80, 100 μmol/L. The minimum concentration of a certain mercuric variety in which bacteria cannot grow was used as the bacteria's MIC of this mercuric variety. LB medium contains sufficient nutrients for bacterial growth; therefore, MIC data measured in LB medium may not reflect the real tolerance of the engineered bacteria. To characterize the MICs of BL21-7 when the nutrition in the medium was poor, a second method was also used to measure the bacterial MICs according to a previously published paper [8]. LB (10 g/L Bacto Tryptone, 5 g/L yeast extract, 10 g/L NaCl, 7 g/L agar, pH 7.0) plates and poor broth nutrient medium (PB) (10 g/L Bacto Tryptone, 5 g/L NaCl, 7 g/L agar, pH 7.0) plates [10] were initially prepared. One clone of BL21-7 on an LB plate was cultured in 5 mL of LB liquid medium containing 50 mg/L kanamycin for 16 h. The 10 μL of this culture grown for 16 h in LB liquid medium was plated on LB plates supplemented with a series of concentrations of each metal variety (the final concentrations of the mercuric varieties in the medium were 3, 5, 10, 20, 40, 60, 80 and 100 μmol/L). Another 10 μL of culture grown for 16 h in LB liquid medium was plated on PB plates supplemented with a series of concentrations of each metal variety (the final concentrations of the mercuric varieties in the medium were 3, 5, 10, 20, 40, 60, 80 and 100 μmol/L). The plates were incubated at 37 °C for 48 h.

Then, the clones on the plates were counted and the minimum concentration of a certain mercuric variety on which the bacteria could not grow was used as the bacteria's MIC of this mercuric variety.

2.6. Hg Content Determination

The Hg content in bacteria was measured following a previously published method [6]. Reagent blanks and spiked control samples were treated as described. The samples were analyzed in an Analyst 200 Perkin Elmer Spectrometer (Perkin Elmer, Waltham, MA, USA) with an MHS-15 Hg-Hydride System (Perkin Elmer, Waltham, MA, USA). Matrix spiked controls were produced by adding 100 ng/mL Hg to *E. coli* cell pellets recovered by centrifugation from 5 mL of LB cultures grown for 16 h without Hg. All samples were measured three times and the average values were used. The Hg accumulation (in μM) was calculated by multiplying the ng/mL (μg/L) value obtained from the instrument by the appropriate dilution factor used to keep the sample within the standard curve range and was then divided by the molecular mass of Hg (200.59 g) in μmol.

2.7. Hg Content Determination Inside Bacterial Cells

One clone of BL21-7 on an LB plate was cultured in 5 mL of LB liquid medium containing 50 mg/L kanamycin for 16 h. Then, 2 mL of this culture was transferred to two groups of conical flasks containing 200 mL of LB liquid medium containing 80 μmol/L $HgCl_2$ and was cultured for 72 h. The bacteria in one group were collected by centrifugation. The Hg content was measured as described in the materials and methods and was named K1. The bacteria in the other group were also centrifuged. The supernatant was discarded and the pellet was thoroughly re-suspended in PBS (phosphate buffer saline) buffer. The mixture was centrifuged and the supernatant was discarded. The pellet was thoroughly re-suspended in PBS buffer once again. This process was repeated three times. Then, the bacteria were thoroughly ground in liquid nitrogen. The abrasive powder was fully suspended in PBS buffer and incubated at room temperature for 1 h. Then, the mixture was centrifuged and the pellet was collected. The Hg in the pellet was measured and named K2. The value of K2 was regarded as the intracellular Hg content. All of the experiments were performed four times and the average values were used.

2.8. Treating Wastewater Using the Engineered Bacteria

One clone each of BL21-7 and BL21-0 on LB plates was cultured in 5 mL of LB liquid medium containing 50 mg/L kanamycin for 16 h. Then, 2 mL of each culture was transferred to 200 mL of LB liquid medium containing 50 mg/L kanamycin and cultured at 37 °C for 5 h. IPTG was then added to the cultures to a final concentration of 0.8 mmol/L. The cultures were cultured at 37 °C for another 12 h. The bacteria were collected by centrifugation at 4 °C and 10,000 *g*. The supernatant was discarded and the pellet was washed with PBS three times. The bacteria suspended in PBS buffer were added 1 L of wastewater. The wastewater was placed in a shaker and cultured at 37 °C and 200 rpm for 24 h. Then, the bacteria were collected by centrifugation at 10,000 *g*. The Hg content in the supernatant was measured. The wastewater sample was obtained from a chlor-alkali plant in Changsha, Hunan province, China. The wastewater was filtered through medium-flow-rate filter paper. Then, the effluent was filtered again through a Millipore membrane with a pore size of 0.45 μm. The characterizations of the waste water was as the following: pH = 7.8, electric conductivity 1760 μs/cm, origin temperature 22 °C, CODCr 760 mg/L, alkalinity 106 mg/L. The content of Ca^{2+}, Mg^{2+}, Na^+, Cl^-, $SO4^{2-}$ was 4.92 mg/L, 0.45 mg/L, 1.18 mg/L, 170 mg/L, 1.8 mg/L, respectively. The total Hg content in the waste water was 0.05 mg/L. To check the effect of the engineered strain, $HgCl_2$ was added into the waste water to the total-Hg content was 0.97 mg/L. All of the experiments were performed four times and the average values were used.

3. Results and Discussion

3.1. Construction of Engineered Bacterial Strain BL21-7

Pseudomonas K-62 is known for its high tolerance to Hg [7,16]. It can transport Hg into the cell by MerT-MerP [16]. The carbon-Hg linkages in organic Hg can be split by MerB1 and MerB2, releasing Hg^{2+} intracellularly [17]. An artificial operon, *P16S-g10-merT-merP-merB1-merB2-ppk-rpsT*, was constructed in this study (Figure 1 and Supplementary Materials S2). *P16S* is one of the strongest constitutive promoters. It is derived from the 5′ UTR of the plastid 16S ribosomal RNA gene [12–14]. The Hg tolerance of bacteria in which the transcription of *mt-1* is promoted by *P16S* was at least six times greater than that of bacteria in which *P16S* was not included [6]. *G10* is a well-known translation enhancer. When placed upstream of a synthetic ribosome-binding site (RBS), there was a 110-fold increase in the translation efficiency of the downstream *lacZ* gene [11]. As a strong terminator, *rpsT* can enhance transcript stability by forming a secondary structure at the 3′ end of the mRNA [15]. The *ppk* gene encodes a key enzyme responsible for synthesizing polyphosphate, which can respond to mercuric ions by chelating reactions and decrease their toxicity to cells [18,19]. The mercuric ions in cells were transferred from the extracellular space by MerT-MerP [16] or from organic Hg by MerB1 and MerB2 [17]. The *E. coli* BL21 (DE3) strain transformed with plasmid pET28a harboring the artificial operon *P16S-g10-merT-merP-merB1-merB2-ppk-rpsT* was named BL21-7.
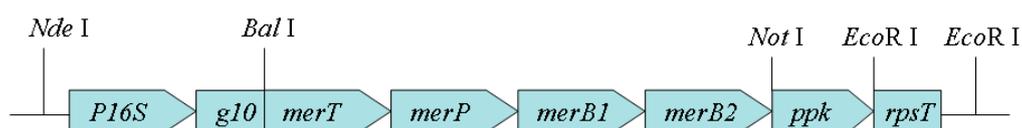
Figure 1. Schematic diagram of the designed artificial operon.

3.2. Transcription of the Artificial Operon P16S-g10-merT-merP-merB1-merB2-ppk-rpsT

BL21-0, BL21-1, BL21-2 and BL21-7 were cultured in LB liquid medium at 37 °C for 16 h. The mRNAs of these bacteria were extracted and qPCR was performed. The cDNA of BL21-0 was used as a control. The results showed that the transcripts of *ppk-rpsT* in BL21-1, BL21-2 and BL21-7 were 1373 $\pm$ 62 copies/ng, 8245 $\pm$ 119 copies/ng and 8326 $\pm$ 141 copies/ng, respectively (Figure 2). The experiment was performed four times. The transcripts of *ppk-rpsT* in BL21-7 were approximately six-fold those in BL21-1 (Figure 2). No transcript was detected in BL21-0. These results demonstrated that the vector p5 (pET28a harboring *P16S-g10-merT-merP-merB1-merB2-ppk-rpsT*) could provide sufficient transcripts. Sufficient transcripts may produce high-abundance protein [6]. The Hg tolerance of BL21-2 was approximately fivefold greater than that of BL21-1 (Figure 3). In BL21-1, the artificial operon *g10-ppk-rpsT* was regulated by the *T7* promoter, while the transcription of the same artificial operon was promoted by *P16S* in BL21-2. This showed that the transcription initiated by *P16S* was stronger than that of the *T7* promoter. The Hg tolerance of BL21-7 (Figure 3) was approximately sevenfold greater than that of the strain transformed with *merR-o/p-merT-merP-merB1-ppk* [20]. This might be due to the strong promoter *P16S*, the translation enhancer *g10* and the terminator *rpsT* in the artificial operon.
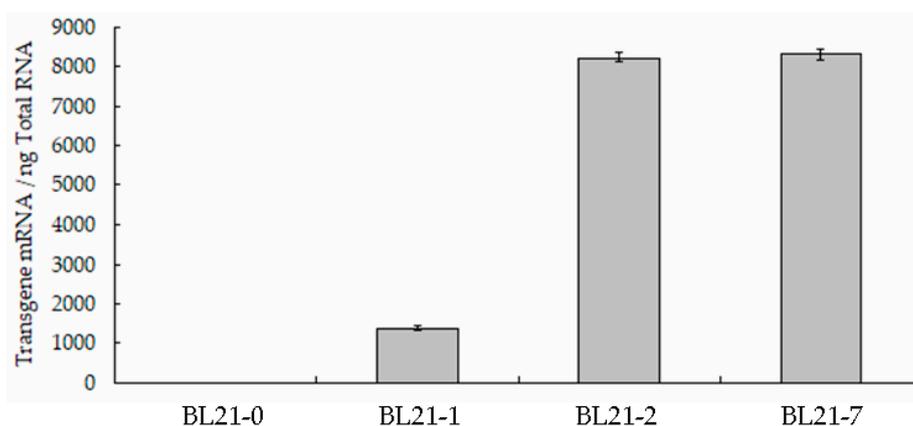
Figure 2. The expression levels of *ppk-rpsT* in different *E. coli* strains. Columns 1-4 represent BL21-0, BL21-1, BL21-2 and BL21-7, respectively.

3.3. Bacterial Strain Tolerance to Hg

All of the bacteria were cultured for 120 h. The OD_{600} values were measured every 2 h. The growth curves of BL21-0, BL21-1, BL21-2 and BL21-7 cultured in liquid LB medium without Hg were S shaped. The growth of these four strains cultured in liquid LB medium containing 5 μmol/L $HgCl_2$ also demonstrated an S shape. However, at the stationary phase, the OD_{600} values of BL21-0 and BL21-1 cultured in the medium containing 5 μmol/L $HgCl_2$ were lower than those corresponding cultures without Hg. The OD_{600} values of BL21-7 cultured in medium containing 5 μmol/L $HgCl_2$ were similar to those of corresponding cultures without Hg. In the media containing 10 μmol/L, 20 μmol/L and 40 μmol/L $HgCl_2$, the growth curves of BL21-7 and BL21-2 still showed an S shape. BL21-0 cultured in LB medium without Hg grew much more quickly than that cultured in LB medium containing $HgCl_2$. The highest concentration of $HgCl_2$ that BL21-0 could withstand was 5 μmol/L (Figure 3). The growth of BL21-0 was inhibited completely in LB medium containing a $HgCl_2$ concentration of 10 μmol/L or more (Figure 3). There were no significant differences between the OD_{600} values of BL21-0 cultured in these media for 16 h and those of BL21-0 cultured in these media for 120 h. BL21-1 was also very sensitive to $HgCl_2$; it could not grow in LB medium containing a $HgCl_2$ concentration of 10 μmol/L or more (Figure 3). After BL21-2 and BL21-7 were cultured in LB liquid medium containing 20 μmol/L $HgCl_2$ for 16 h, their OD_{600} values were 1.1 ± 0.05 and 1.2 ± 0.05, respectively. They exhibited good tolerance to 20 μmol/L $HgCl_2$ (Figure 3). After being cultured for 120 h, BL21-7 cultured in LB containing 20 μmol/L $HgCl_2$ had similar OD_{600} values to BL21-7 cultured in LB without Hg. The bacterial strain tolerance to $HgCl_2$ can be ordered from the strongest to the weakest as follows: BL21-7, BL21-2, BL21-1 and BL21-0.
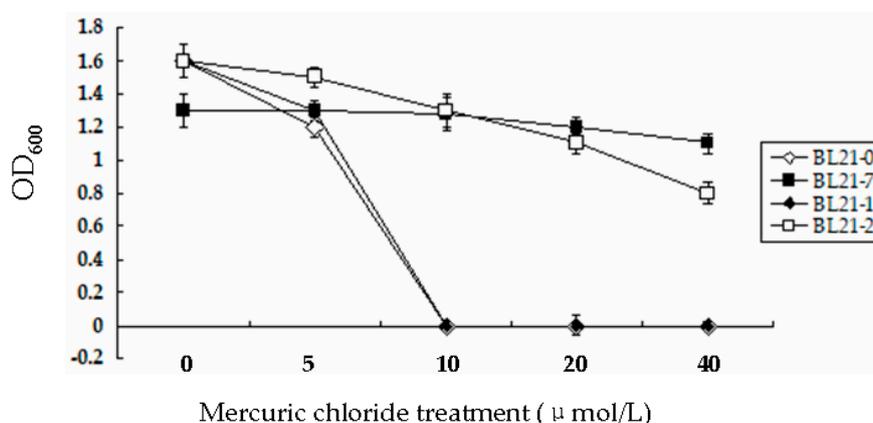
Figure 3. *E. coli* strains' tolerance to $HgCl_2$. The OD_{600} values were measured after the strains were cultured in LB liquid medium containing $HgCl_2$ for 120 h.

To identify whether BL21-7 displayed good tolerance to organic Hg, the engineered bacterial strain was cultured in a series of LB media containing different quantities of MMC or PMC. All of the bacteria were cultured for 120 h. The OD_{600} values were measured every 2 h. The growth curves of BL21-0 and BL21-7 cultured in liquid LB medium without Hg showed an S shape. BL21-0 grew well in liquid LB medium containing 5 μmol/L MMC. However, it did not grow in LB medium containing 5 μmol/L PMC or 10 μmol/L MMC. After BL21-7 was cultured in LB liquid medium containing 10 μmol/L MMC for 16 h, the OD_{600} value of the culture was still approximately 1.4 ± 0.08. This bacterial strain grew well in LB containing 20 μmol/L MMC, 40 μmol/L MMC, 20 μmol/L PMC or 40 μmol/L PMC (Figure 4).
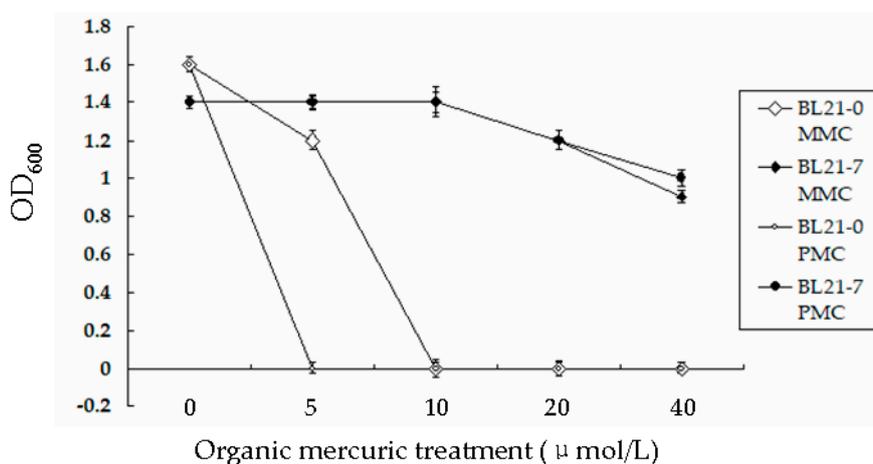
Figure 4. *E. coli* strains' tolerance to organic Hg. The OD_{600} values were measured after the strains were cultured in LB liquid medium containing MMC or PMC for 120 h.

To further identify the engineered strain's tolerance to Hg, its MICs of $HgCl_2$, PMC and MMC were measured. A series of conical flasks were filled with 200 mL of fresh LB liquid medium. A stock solution of $HgCl_2$ (0.1 mol/L) was initially prepared and a series of $HgCl_2$ stock solutions were added to the medium to final concentrations of 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μmol/L. The OD_{600} values of the cultures were measured every h. The minimum Hg concentration of LB liquid medium in which the bacteria could not grow was used as the MIC. The bacteria's MICs for MMC and PMC were measured following the same method. All of the experiments were performed four times and the average values were used. The results showed that the growth curves of BL21-7 cultured in LB containing 0 μmol/L, 5 μmol/L and 10 μmol/L $HgCl_2$ were very similar (Figure 5a). As shown in Figure 5, they could not be distinguished. The growth curves of BL21-7 cultured in LB containing 0 μmol/L, 5 μmol/L and 10 μmol/L MMC were also very similar (Figure 5b). For BL21-7, the MICs of Hg^{2+}, MMC and PMC were 100 μmol/L, 60 μmol/L and 80 μmol/L, respectively (Figure 5a–c). BL21-7 could remove approximately 80% of the Hg^{2+} in the medium when the Hg^{2+} concentration was 80 μmol/L (Supplementary Materials Figure S3a). BL21-7 could effectively accumulate MMC and PMC when the concentrations of MMC and PMC were approximately 40 μmol/L and 60 μmol/L, respectively (Supplementary Materials Figure S3b,c). For BL21-7 on LB plates, the MICs of $HgCl_2$, MMC and PMC were 100 μmol/L, 60 μmol/L and 80 μmol/L, respectively. These MICs were consistent with the measured MIC of the Hg remaining in the supernatant. To characterize the MICs of BL21-7 when the nutrition in the medium was poor, this strain was cultured on PB plates containing different Hg derivatives. The results showed that for BL21-7 on PB plates, the MICs of $HgCl_2$, MMC and PMC were 30 μmol/L, 20 μmol/L and 30 μmol/L, respectively.

(**a**)

(**b**)

(**c**)
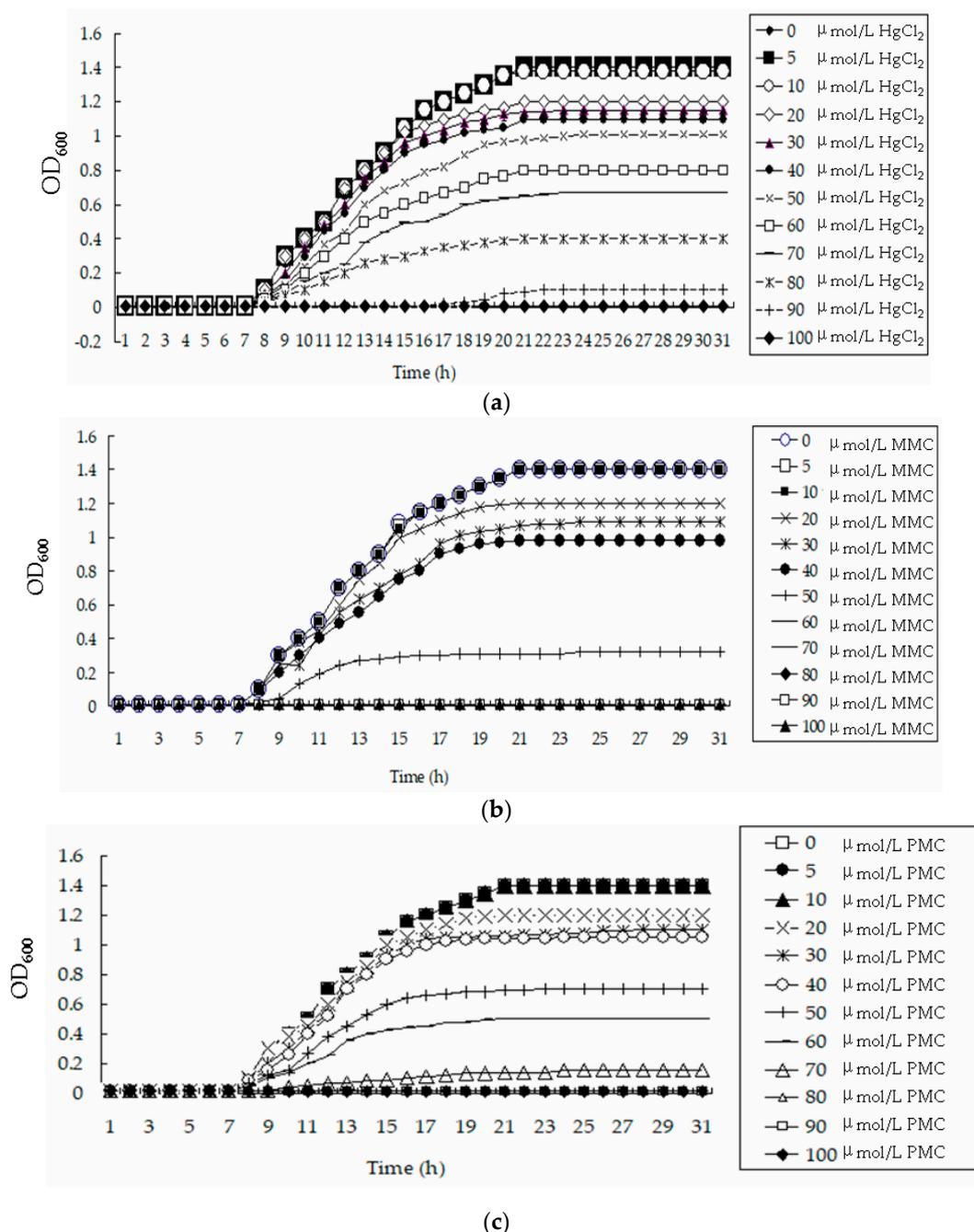
Figure 5. Growth curves of BL21-7 cultured in LB liquid medium containing mercurial derivatives. (**a**) shows BL21-7 cultured in LB liquid medium containing $HgCl_2$. (**b**) represents BL21-7 cultured in the presence of MMC. (**c**) shows BL21-7 cultured in the presence of PMC.

An engineered bacterial strain, MSR33 has been reported, with MICs of $HgCl_2$ and MMC of 120 μmol/L and 80 μmol/L, respectively [8]. CM111 and HG3 also had high $HgCl_2$ MICs [10]. Mt-1 could effectively accumulate Hg from medium containing 120 μmol/L $HgCl_2$ [6]. These bacteria all had higher MICs than that of BL21-7. However, the mechanism of MSR33 for removing Hg involves volatilization, which releases Hg into the air and pollutes the environment. The tolerance of CM111 and HG3 to $HgCl_2$ was thought to not be due to Hg(0) volatilization [10]. However, the detailed mechanisms by which these two strains tolerated $HgCl_2$ remained unclear. Although mt-1 had a high tolerance to $HgCl_2$, it remains unknown whether it can accumulate organic Hg. Comparatively, BL21-7 not only exhibited high MICs of $HgCl_2$, MMC and PMC (Figure 5) but could also store Hg

in the cell (Supplementary Materials S3). BL21-7 not only accumulated Hg^{2+} but also effectively accumulated organic Hg (MMC and PMC) (Supplementary Materials S3). Kiyono et al. constructed an engineered bacterial strain harboring the pMKB18 plasmid, which contained *merT-merP-merB1-ppk* [9]. After the engineered bacteria were cultured in LB medium containing 80 μmol/L $HgCl_2$ for 5 h, the Hg remaining in the medium was nearly 40 μmol/L [9]. However, after BL21-7 was cultured in LB medium containing 80 μmol/L $HgCl_2$ for 5 h, the Hg remaining in the medium was only approximately 25 ± 4 μmol/L (Supplementary Materials Figure S3a). BL21-7 could also remove more phenylmercury from the medium than *E. coli* harboring pMKB18 (Supplementary Materials Figure S3b) [9]. These findings demonstrated that BL21-7 had greater Hg-accumulation capacity than *E. coli* harboring pMKB18.

3.4. Accumulation of Hg Derivatives by BL21-7 Cultured in LB Medium

To further identify whether the bacteria could accumulate more Hg when they were cultured for a sufficient length of time, Hg in bacteria was measured after the bacteria were cultured in media containing mercuric varieties for 72 h. When bacterial BL21-7 accumulated Hg from the liquid medium, Hg could attach to the cell wall and not enter the bacterial cell. The data measured above included Hg attached to the cell wall and Hg in the bacterial cells. To measure the Hg content inside bacterial cells, we measured the total Hg content and the Hg attached to the cell wall. The difference between the total Hg content and the Hg attached to the cell wall was regarded as the intracellular Hg content. The results showed that after BL21-7 was cultured in LB medium containing 80 μmol/L $HgCl_2$ for 72 h, the Hg content in the bacterial pellet was 70.5 ± 1.5 μmol/L (Figure 6). The intracellular Hg content was 56.2 ± 1.3 μmol/L (Figure 6). After BL21-7 was cultured in LB medium containing 40 μmol/L MMC or 60 μmol/L PMC for 72 h, the Hg content in the bacterial pellets was 33.5 ± 3.2 μmol/L and 45.3 ± 3.7 μmol/L, respectively (Figure 6). The intracellular Hg content was 28.3 ± 3.0 μmol/L and 36.5 ± 3.1 μmol/L, respectively (Figure 6). These results demonstrated that the engineered bacteria could efficiently accumulate $HgCl_2$, MMC and PMC. This bacterial strain not only accumulated mercuric ions in the medium but also effectively accumulated organic Hg (PMC and MMC).
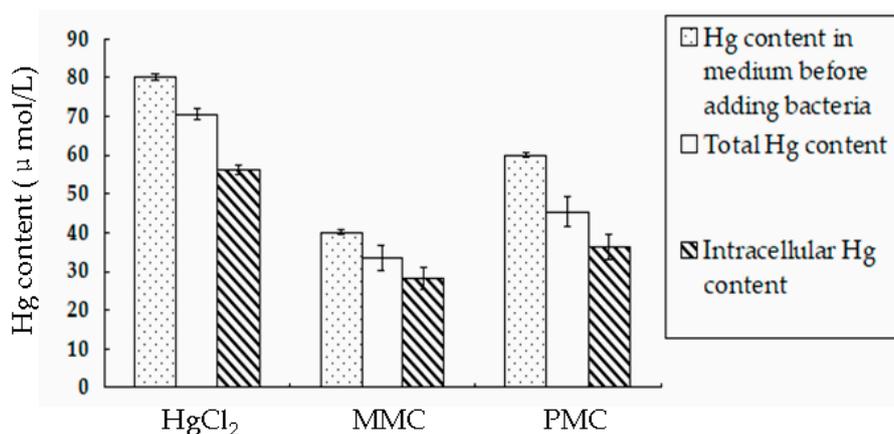
Figure 6. Hg content in BL21-7 bacteria cultured in LB liquid medium containing mercurial derivatives.

3.5. Effects of Other Metals on the Accumulation of Hg by BL21-7

Cd^{2+}, Pb^{2+} and Cu^{2} are always found at Hg-polluted sites. To characterize whether these metals would affect the engineered bacterial strain when it was used to remove Hg from a medium, the MICs of BL21-7 for Cd^{2+}, Pb^{2+} and Cu^{2+} were also measured. All of the experiments were performed four times and the average values were used. The results for BL21-7 on LB plates showed that the MICs for Cd^{2+}, Pb^{2+} and Cu^{2+} were 13 μmol/L, 12 μmol/L and 22 μmol/L, respectively. After BL21-7 was cultured in LB liquid medium containing 10 μmol/L mercurial derivatives ($HgCl_2$, MMC or PMC) without other metals for 5 h, the concentrations of the Hg removed from the medium were

8.80 μmol/L, 5.47 μmol/L and 7.80 μmol/L, respectively. After the engineered bacteria were cultured in LB liquid medium containing 10 μmol/L mercurial derivatives ($HgCl_2$, MMC or PMC) in the presence of 10 μmol/L Cd^{2+} for 5 h, the concentrations of the Hg removed from the medium were 8.66 μmol/L, 5.53 μmol/L and 7.76 μmol/L, respectively (Table 3). No significant differences were found between the Hg removed from the medium containing Cd^{2+} and the Hg from the medium without Cd^{2+}. Similar results were obtained after BL21-7 was cultured in medium containing 10 μmol/L mercurial derivatives ($HgCl_2$, MMC or PMC) in the presence of 10 μmol/L Pb^{2+} or Cu^{2+} (Table 3). After BL21-7 was cultured in LB medium containing 10 μmol/L mercurial derivatives ($HgCl_2$, MMC or PMC) in the presence of 10 μmol/L Cd^{2+}, 10 μmol/L Pb^{2+} and 10 μmol/L Cu^{2+} for 5 h, the values of the Hg removed were the highest among the four treatments. However, when the standard deviations were considered, the differences between the values of Hg removed from the medium without other metals and the values of Hg removed from the medium containing 10 μmol/L Cd^{2+} (or the other three treatments) were not significant. These findings demonstrated that in the presence of 10 μmol/L Cd^{2+}, Pb^{2+} or Cu^{2+}, the removal of mercurial derivatives by BL21-7 was not affected (Table 3).

Table 3. Mercury removed from LB medium in which BL21-7 was cultured for 5 h in the presence of other metals.

Metals	$HgCl_2$ Removed (μmol/L)	MMC Removed (μmol/L)	PMC Removed (μmol/L)
None	8.80 ± 0.16	5.47 ± 0.28	7.80 ± 0.17
Cd^{2+}	8.66 ± 0.15	5.53 ± 0.23	7.76 ± 0.15
Pb^{2+}	8.85 ± 0.09	5.41 ± 0.25	7.73 ± 0.18
Cu^{2+}	8.95 ± 0.12	5.44 ± 0.26	7.83 ± 0.17
$Cd^{2+} + Pb^{2+} + Cu^{2+}$	8.98 ± 0.19	5.59 ± 0.27	7.85 ± 0.19

3.6. Effects of pH and Temperature on the Accumulation of Hg by BL21-7

To characterize the effects of pH on the ability of the engineered bacteria to remove Hg from the environment, BL21-7 was cultured in LB liquid medium overnight at 37 °C. One microliter of this culture was transferred to 100 mL of nutrition-poor PB medium (pH = 5, 6, 7, 8 or 9) containing 20 μmol/L $HgCl_2$ and was cultured continually. The OD_{600} values of the cultures were measured every two h. After the cultures entered stationary phase, the Hg contents in the bacteria and medium were measured. The results showed that all of the growth curves of the cultures showed an S shape. No significant differences were found among the growth curves of cultures at pH = 5, 7 and 8 (Figure 7). For example, after BL21-7 was cultured for 30 h, the OD_{600} values of the cultures (pH = 5, 7 and 8) were 1.21 ± 0.03, 1.22 ± 0.03 and 1.2 ± 0.03, respectively. Among the five PB media (pH = 5, 6, 7, 8 and 9), the OD_{600} values of BL21-7 cultured in PB at pH = 6 were the highest in stationary phase. The engineered bacteria could also grow in PB at pH = 9. However, they grew very slowly (Figure 7). The final OD_{600} of the culture at pH = 9 was also the lowest among the five treatments. After BL21-7 was cultured for 40 h, the Hg contents in the supernatant of the PB media (pH = 5, 6, 7, 8 and 9) containing 20 μmol/L $HgCl_2$ were 6.1 ± 0.10 μmol/L, 5.2 ± 0.11 μmol/L, 5.9 ± 0.12 μmol/L, 7.6 ± 0.12 μmol/L and 12.5 ± 0.11 μmol/L, respectively. The Hg content in the supernatant of the PB at pH = 6 containing 20 μmol/L $HgCl_2$ was the lowest among the five treatments. The engineered bacterial strain accumulated the most Hg in the liquid medium at a pH of 6 and it worked well in the media with pH values from 5 to 8.

To characterize the effects of temperature on the engineered strain's growth and accumulation of Hg, BL21-7 was cultured in LB liquid medium overnight at 37 °C. One microliter of this culture was transferred to 100 mL of nutrition-poor PB medium containing 20 μmol/L $HgCl_2$ and was cultured at 25 °C, 30 °C and 37 °C. The OD_{600} values and Hg content were measured as described above. The results showed that although BL21-7 grew more slowly at 25 °C than at 30 °C or 37 °C, the OD_{600} values of these three treatments were similar at stationary phase (Figure 8). For example, although

the OD_{600} values of the cultures (T = 25 °C, 30 °C and 37 °C) at 20 h were 0.6 ± 0.04, 1.1 ± 0.04 and 1.2 ± 0.03, respectively, the OD_{600} values of the same cultures at 34 h were all 1.3 ± 0.03. After BL21-7 was cultured for 20 h, the Hg contents in the supernatant of the PB medium (T = 25 °C, 30 °C and 37 °C) containing 20 μmol/L $HgCl_2$ were 11.2 ± 0.12 μmol/L, 8.2 ± 0.15 μmol/L and 6.5 ± 0.11 μmol/L, respectively. When BL21-7 was cultured for 34 h, the Hg contents in the supernatant of the PB medium (T = 25 °C, 30 °C and 37 °C) containing 20 μmol/L $HgCl_2$ were 6.1 ± 0.11 μmol/L, 6.2 ± 0.15 μmol/L and 6.0 ± 0.12 μmol/L, respectively. At stationary phase, the Hg contents in the supernatant of the PB medium (T = 25 °C, 30 °C and 37 °C) containing 20 μmol/L $HgCl_2$ were similar. These data indicate that BL21-7 functions well at temperatures from 25 °C to 37 °C.
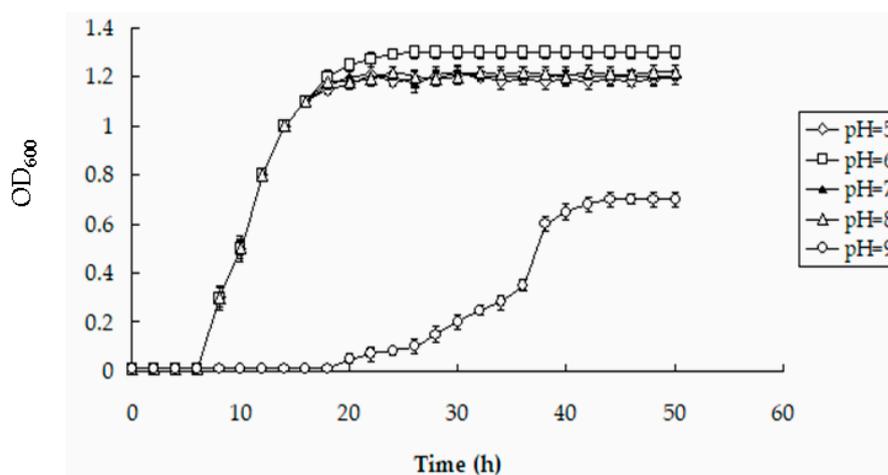
Figure 7. Growth curves of BL21-7 cultured in PB media at different pH values.
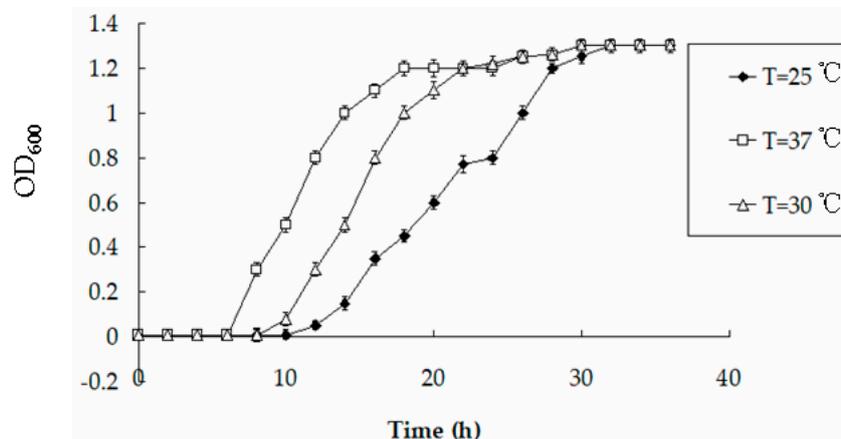
Figure 8. Growth curves of BL21-7 cultured at different temperatures.

3.7. Hg in Wastewater Can Be Removed by BL21-7

To identify whether the engineered bacteria BL21-7 could be used to remove Hg from wastewater, 200 mL of BL21-7 bacteria and BL21-0 bacteria (*E. coli* BL21 harboring the empty vector pET28a) were treated as described in Methods and were added to 1 L of wastewater. The cultures were shaken at 37 °C and 200 rpm for 24 h. Then, the bacteria were collected and the Hg content in the wastewater was measured. The results showed that before the wastewater was treated using the engineered bacteria, the Hg content in the wastewater samples was 4.85 ± 0.31 μmol/L (Figure 9). After the engineered bacteria BL21-7 were added to the wastewater and shaken at 37 °C for 24 h, the Hg content in the wastewater was reduced to 2.73 ± 0.15 μmol/L (Figure 9). Approximately 43.7% of the Hg in the wastewater had been removed. However, after BL21-0 was added to the wastewater and shaken at 37 °C for 24 h, the Hg content in the wastewater was 4.8 ± 0.27 μmol/L (Figure 9).

This result demonstrates that the engineered bacteria BL21-7 can accumulate Hg in wastewater. Only bacterial cells were added to the wastewater in our experiments. If substances containing sufficient nutrients were added to the wastewater with the bacterial cells, the effect on Hg accumulation might be more significant.
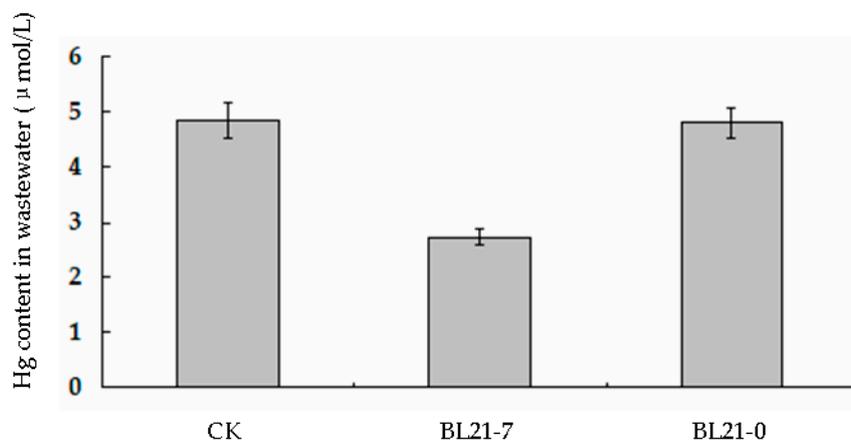
Figure 9. Hg content in wastewater. CK indicates the Hg content in the wastewater sample without engineered bacteria. BL21-7 indicates the Hg content in the wastewater treated with BL21-7. BL21-0 indicates the Hg content in the wastewater treated with BL21-0.

3.8. The Roles of the Genes in the Artificial Operon

To characterize the effects of the different genes in the artificial operon on Hg accumulation, strains harboring different mer and *ppk* genes were constructed. After the strains were cultured in medium containing Hg for a certain time span, the MRMs were measured. For Hg^{2+} accumulation, the MRMs of BL21-B1, BL21-B2 and BL21-B1B2 were similar to that of BL21-0 (Table 4). The MRM of BL21-P was lower than those of the strains above. BL21-P accumulated more Hg than the strains described above. MerP can bind to Hg^{2+} and plays a role as a scavenger to protect other periplasmic and membrane proteins from binding to Hg^{2+} [21]. BL21-T transports more Hg^{2+} than BL21-P. MerT is responsible for transporting Hg^{2+} into cells [16]. The MRM of BL21-7 was the lowest among all the strains, indicating that BL21-7 was the most effective strain for accumulating Hg^{2+}.

Table 4. Mercury remaining in the medium after strains were cultured in LB medium.

Strains	HgCl$_2$ (µmol/L)					MMC (µmol/L)					PMC (µmol/L)				
	0 h	5 h	10 h	15 h	20 h	0 h	5 h	10 h	15 h	20 h	0 h	5 h	10 h	15 h	20 h
BL21-T	20.0	17.1	16.3	15.2	15.0	20.0	19.8	19.5	19.3	19.0	20.0	19.9	19.9	19.8	19.6
BL21-B1	20.0	19.8	19.8	19.8	19.4	20.0	19.8	19.2	18.5	18.1	20.0	18.2	17.5	16.3	15.5
BL21-B2	20.0	19.8	19.8	19.7	19.6	20.0	19.3	18.9	18.8	18.8	20.0	18.2	17.3	16.5	16.2
BL21-B1B2	20.0	19.9	19.8	19.5	19.2	20.0	19.5	18.5	18.0	17.5	20.0	17.4	16.5	15.3	15.1
BL21-TP	20.0	14.8	13.2	11.5	11.1	20.0	19.9	19.7	19.5	19.5	20.0	19.9	19.9	19.8	19.5
BL21-P	20.0	19.6	18.5	18.3	18.0	20.0	19.9	19.8	19.8	19.5	20.0	19.8	19.7	19.5	19.5
BL21-0	20.0	19.9	19.8	19.7	19.2	20.0	19.8	19.9	19.6	19.6	20.0	19.8	19.7	19.5	19.3
BL21-1	20.0	19.8	19.7	19.9	19.5	20.0	19.9	19.5	19.4	19.4	20.0	19.9	19.8	19.5	19.2
BL21-2	20.0	12.5	11.2	10.0	9.5	20.0	19.8	19.9	19.6	19.6	20.0	19.8	19.8	19.6	19.5
BL21-B1B2K	20.0	13.1	12.5	11.3	10.0	20.0	16.1	15.3	14.2	14.1	20.0	10.2	8.5	6.8	6.1
BL21-TPK	20.0	7.9	4.5	3.8	2.2	20.0	19.8	19.6	19.5	19.3	20.0	19.9	19.8	19.8	19.5
BL21-7	20.0	6.8	3.7	2.5	1.1	20.0	6.7	3.2	1.4	1.1	20.0	5.2	2.3	1.5	1.0
BL21-TPB1B2	20.0	14.6	13.2	12.3	12.2	20.0	19.5	19.2	18.3	17.2	20.0	17.5	17.1	15.2	15.1

For MMC accumulation, the MRM of BL21-7 was much lower than that of BL21-0 (Table 4), indicating that BL21-7 could also effectively accumulate MMC. The MRMs of BL21-T, BL21-P, BL21-TP, BL21-TPK and BL21-0 were similar, demonstrating that MerT and MerP did not participate in transporting MMC. The MRM of BL21-TPK was similar to that of BL21-TP, indicating that polyphosphate could not bind to MMC. The MRMs of BL21-B1 and BL21-B2 were lower than that of

BL21-0, suggesting that MerB1 and MerB2 could also catalyze the splitting of the carbon-Hg bond in MMC. BL21-7 was the best strain for MMC removal. After BL21-7 was cultured in LB medium containing 20 μmol/L MMC for 15 h, the MRM was only approximately 1.4 μmol/L (Table 4).

For PMC accumulation, the MRMs of BL21-B1 and BL21-B2 were lower than those of BL21-T, BL21-TP and BL21-TPK, suggesting that MerB1 and MerB2 could promote PMC accumulation. This result could be due to their increasing the host cell's tolerance to PMC, as MerB1 and MerB2 can catalyze the splitting of the carbon-Hg bond [22–24]. The MRM of BL21-7 was the lowest among the strains. The second lowest MRM was BL21-B1B2K.

Although *Pseudomonas* K-62 has been shown to have approximately 1000-fold higher resistance to phenylmercury than *E. coli* [25], its biosafety had not been studied. *E. coli* BL21(DE3) was derived from normal commensals of the human gut [26] and is not harmful to humans. Therefore, we selected *E. coli* BL21(DE3), not *Pseudomonas* K-62, for transforming and constructing the engineered bacterial strain. The genome of *E. coli* BL21(DE3) has been sequenced and published [26]. However, although *merB* genes had been found in *E. coli* R831b and *E. coli* AAD3 [27], we were unable to find any mer genes in the *E. coli* BL21(DE3) genome. Compared to that of *Pseudomonas* K-62, the Hg-resistance phenotype of *E. coli* BL21(DE3) is negligible. To better understand the engineered bacteria's ability to accumulate Hg, a mechanism for organic and inorganic Hg detoxification by BL21-7's artificial operon was proposed (Figure 10). According to the proposed mechanism, in the artificial operon, both *P16S* and *g10* can increase the expression levels of downstream genes [11,12]. *RpsT* can prevent the transcripts from being hydrolyzed [6]. This construct can result in sufficient transcription and protein accumulation for Hg tolerance and Hg accumulation. Phenylmercury and Hg^{2+} are transported into the cell periplasm by MerP [21]. The phenylmercury and Hg^{2+} in the periplasm are transported into the cell by MerT [8]. MerP can bind to Hg^{2+} and retain it in the periplasm [21]. However, high levels of phenylmercury in a cell can harm the cell. The phenylmercury tolerance of BL21-TP was lower than that of BL21-0. Methylmercury is transported into the cell by unknown means, as the MerT-MerP system does not transport methylmercury [7]. The methylmercury tolerance and methylmercury accumulation of BL21-T, BL21-P and BL21-TP were similar to those of BL21-0. Being catalyzed by MerB1 and MerB2, the carbon-Hg bond in phenylmercury or methylmercury is split and Hg^{2+} is released [22–24]. The toxicity of Hg^{2+} is lower than that of organic Hg. The tolerance to phenylmercury and methylmercury of BL21-TPB1B2 are higher than those of BL21-0. After *ppk* is overexpressed, a significant amount of polyP is produced. Hg^{2+} binds to polyP, forming the chelate polyP-Hg^{2+} [9]. The toxicity of polyP-Hg^{2+} is lower than that of Hg^{2+}. Thus, the Hg tolerance and Hg accumulation of bacterial strains harboring *ppk* are higher than those of strains without the *ppk* gene.
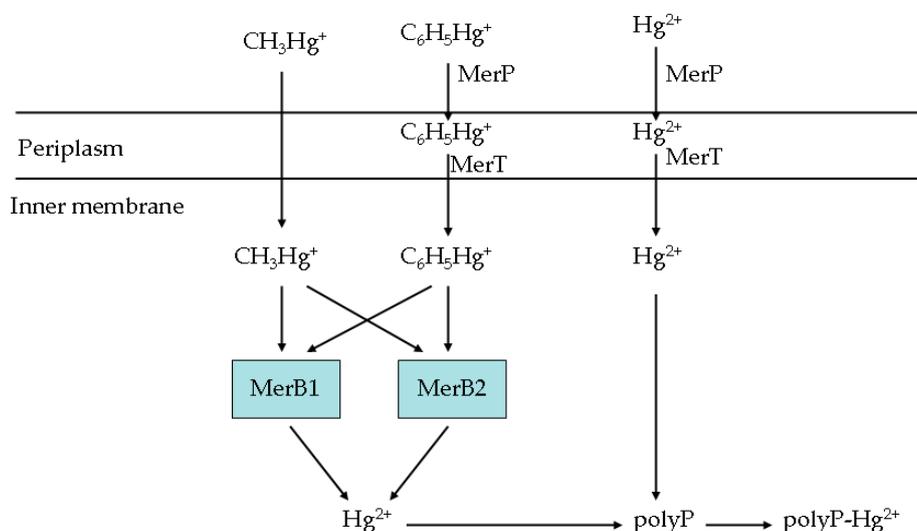
Figure 10. Schematic mechanism proposed for organic and inorganic Hg detoxification by the constructed operon of BL21-7.

4. Conclusions

The construction of an engineered bacterial strain, *E. coli* BL21-7, was reported in this study. The constructed plasmid contained a strong promoter, *P16S*, a translation enhancer, *g10*, a strong terminator, *rpsT*, *Pseudomonas* K-62 genes *merT*, *merP*, *merB1* and *merB2* and the *Klebsiella aerogenes* polyphosphate kinase gene *ppk*. This construct provided sufficient transcripts for Hg tolerance and Hg accumulation. A mechanism for Hg detoxification by BL21-7 was proposed. This bacterial strain did not release Hg into the environment. It could accumulate both inorganic and organic Hg. When the engineered bacterial strain BL21-7 was cultured in LB liquid medium containing 10 µmol/L mercurial derivatives (Hg^{2+}, PMC or MMC) in the presence of 10 µmol/L Cd^{2+}, 10 µmol/L Pb^{2+} or 10 µmol/L Cu^{2+}, the removal of mercurial derivatives by BL21-7 was not affected. After BL21-7 bacterial samples were added to wastewater, the Hg content in the wastewater could be reduced significantly. These characteristics make BL21-7 a good candidate for the removal of Hg from wastewater.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/8/9/1572/s1, S1: The sequence of the artificial operon *P16S-g10-merT-merP-merB1-merB2-ppk-rpsT*, S2: Construction of artificial operons and respective bacterial strains, S3: Hg remaining in medium after BL21-7 was cultured in LB liquid medium containing mercurial derivatives.

Author Contributions: S.C. and Z.W. did data curation. They were equal contributors. W.S. did data curation. H.S. validated the data and wrote the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jan, A.T.; Murtaza, I.; Ali, A.; Mohd, Q.; Haq, R. Mercury pollution: An emerging problem and potential bacterial remediation strategies. *World J. Microbiol. Biotechnol.* **2009**, *25*, 1529–1537. [CrossRef]
2. U.S. Environmental Protection Agency (US EPA). EPA's Roadmap for Mercury. July 2006. Available online: http://www.epa.gov/mercury/roadmap.htm (accessed on 26 October 2007).
3. U.S. Environmental Protection Agency (US EPA) and Environment Canada. Update: Binational Toxics Strategy—Mercury Sources and Regulations. 1999. Available online: http://www.epa.gov/bns/mercury/stephg.html (accessed on 26 October 2007).
4. U.S. Environmental Protection Agency (US EPA). Great Lakes Pollution Prevention and Toxics Strategy. Background Information on Mercury Sources and Regulations. 2005. Available online: http://www.epa.gov/grtlakes/bnsdocs/mercsrce/mercsrce.html (accessed on 26 October 2007).
5. Cachada, A.M.; Rodrigues, S.; Mieiro, C.; Ferreira da Silva, E.; Pereira, E.; Duarte, A.C. Controlling factors and environmental implications of mercury contamination in urban and agricultural soils under a long-term influence of a chlor-alkali plant in the North–West Portugal. *Environ. Geol.* **2009**, *57*, 91–98. [CrossRef]
6. Ruiz, O.N.; Alvarez, D.; Gonzalez-Ruiz, G.; Torres, C. Characterization of mercury bioremediation by transgenic bacteria expressing metallothionein and polyphosphate kinase. *BMC Biotechnol.* **2011**, *11*, 1–8. [CrossRef] [PubMed]
7. Kiyono, M.; Omura, T.; Fujimori, H.; Pan-Hou, H. Lack of involvement of merT and merP in methylmercury transport in mercury resistant Pseudomonas K-62. *FEMS Microbiol. Lett.* **1995**, *128*, 301–306. [CrossRef] [PubMed]
8. Rojas, L.A.; Yanez, C.; Gonzalez, M.; Lobos, S.; Smalla, K.; Seeger, M. Characterization of the metabolically modified heavy metal-resistant Cupriavidus metallidurans strain MSR33 generated for mercury bioremediation. *PLoS ONE* **2011**, *6*, e17555. [CrossRef] [PubMed]
9. Kiyono, M.; Omura, H.; Omura, T.; Murata, S.; Pan-Hou, H. Removal of inorganic and organic mercurials by immobilized bacteria having mer-ppk fusion plasmids. *Appl. Microbiol. Biotechnol.* **2003**, *62*, 274–278. [CrossRef] [PubMed]
10. Francois, F.; Lombard, C.; Guigner, J.; Soreau, P.; Brian-Jaisson, F.; Martino, G.; Vandervennet, M.; Garcia, D.; Molinier, A.; Pignol, D.; et al. Isolation and characterization of environmental bacteria capable of extracellular biosorption of mercury. *Appl. Environ. Microbiol.* **2011**, *78*, 1097–1106. [CrossRef] [PubMed]

11. Olins, P.O.; Rangwala, S.H. A novel sequence element derived from bacteriophage T7 mRNA acts as an enhancer of translation of the lacZ gene in *Escherichia coli*. *J. Biol. Chem.* **1989**, *264*, 16973–16976. [PubMed]

12. Dennis, P.P.; Ehrenberg, M.; Fange, D.; Bremer, H. Varying rate of RNA chain elongation during rrn transcription in *Escherichia coli*. *J. Bacteriol.* **2009**, *191*, 3740–3746. [CrossRef] [PubMed]

13. Newell, C.A.; Birch-Machin, I.; Hibberd, J.M.; Gray, J.C. Expression of green fluorescent protein from bacterial and plastid promoters in tobacco chloroplasts. *Transgenic Res.* **2003**, *12*, 631–634. [CrossRef] [PubMed]

14. Kuroda, H.; Maliga, P. Complementarity of the 16S rRNA penultimate stem with sequences downstream of the AUG destabilizes the plastid mRNAs. *Nucleic Acids Res.* **2001**, *29*, 970–975. [CrossRef] [PubMed]

15. Shiina, T.; Allison, L.; Maliga, P. RbcL transcript levels in tobacco plastids are independent of light: Reduced dark transcription rate is compensated by increased mRNA stability. *Plant Cell* **1998**, *10*, 1713–1722. [CrossRef] [PubMed]

16. Silver, S.; Phung, L.T. Bacterial heavy metal resistance: New surprises. *Annu. Rev. Microbiol.* **1996**, *50*, 753–789. [CrossRef] [PubMed]

17. Kiyono, M.; Pan-Hou, H. Genetic engineering of bacteria for environmental remediation of mercury. *J. Health Sci.* **2006**, *52*, 199–204. [CrossRef]

18. Pan-Hou, H.; Kiyono, M.; Kawase, T.; Omura, T.; Endo, G. Evaluation of ppk-specified polyphosphate as a mercury remedial tool. *Biol. Pharm. Bull.* **2001**, *24*, 1423–1426. [CrossRef] [PubMed]

19. Kornberg, A. Inorganic polyphosphate: Toward making a forgotten polymer unforgettable. *J. Bacteriol.* **1995**, *177*, 491–496. [CrossRef] [PubMed]

20. Pan-Hou, H.; Kuyono, M.; Omura, H.; Omura, T.; Endo, G. Polyphosphate produced in recombinant *Escherichia coli* confers mercury resistance. *FEMS Microbiol. Lett.* **2002**, *207*, 159–164. [CrossRef] [PubMed]

21. Sone, Y.; Nakamura, R.; Pan-Hou, H.; Itoh, T.; Kiyono, M. Role of MerC, MerE, MerF, MerT, and/or MerP in resistance to mercurials and the transport of mercurials in *Escherichia coli*. *Biol. Pharm. Bull.* **2013**, *36*, 1835–1841. [CrossRef] [PubMed]

22. Misra, T.K. Bacterial resistances to inorganic mercury salts and organomercurials. *Plasmid* **1992**, *27*, 1, 4–16. [CrossRef]

23. Tezuka, T.; Tonomura, K. Purification and properties of an enzyme catalyzing the splitting of carbon-mercury linkages from mercury-resistant Pseudomonas K-62 strain. *J. Biochem.* **1976**, *80*, 79–87. [CrossRef] [PubMed]

24. Tezuka, T.; Tonomura, K. Purification and properties of a second enzyme catalyzing the splitting of carbon-mercury linkages from mercury-resistant Pseudomonas K-62. *J. Bacteriol.* **1978**, *135*, 138–143. [PubMed]

25. Tonomura, K.; Maeda, K.; Futai, F.; Nakagami, T.; Yamada, M. Stimulative vaporization of phenylmercuric acetate by mercury-resistant bacteria. *Nature* **1968**, *217*, 644–646. [CrossRef] [PubMed]

26. Studier, F.W.; Daegelen, P.; Lenski, R.E.; Maslov, S.; Kim, J.F. Understanding the differences between genome sequences of *Escherichia coli* B strains REL606 and BL21(DE3) and comparision of the *E. coli* B and K-12 genomes. *J. Mol. Biol.* **2009**, *394*, 653–680. [CrossRef] [PubMed]

27. Barkay, T.; Wagner-Dobler, I. Microbial transformations of mercury: Potentials, challenges and achievements in controlling mercury toxicity in the environment. *Adv. Appl. Microbiol.* **2005**, *57*, 1–52. [PubMed]