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ENVIRONMENTAL BIOTECHNOLOGY

Characterization of a marine-isolated mercury-resistant *Pseudomonas putida* strain SP1 and its potential application in marine mercury reduction

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Abstract The *Pseudomonas putida* strain SP1 was isolated from marine environment and was found to be resistant to 280 μM $HgCl_2$. SP1 was also highly resistant to other metals, including $CdCl_2$, $CoCl_2$, $CrCl_3$, $CuCl_2$, $PbCl_2$, and $ZnSO_4$, and the antibiotics ampicillin (Ap), kanamycin (Kn), chloramphenicol (Cm), and tetracycline (Tc). *mer* operon, possessed by most mercury-resistant bacteria, and other diverse types of resistant determinants were all located on the bacterial chromosome. Cold vapor atomic absorption spectrometry and a volatilization test indicated that the isolated *P. putida* SP1 was able to volatilize almost 100% of the total mercury it was exposed to and could potentially be used for bioremediation in marine environments. The optimal pH for the growth of *P. putida* SP1 in the presence of $HgCl_2$ and the removal of $HgCl_2$ by *P. putida* SP1 was between 8.0 and 9.0, whereas the optimal pH for the expression of *merA*, the mercuric reductase enzyme in *mer* operon that reduces reactive Hg^{2+} to volatile and relatively inert monoatomic Hg^0 vapor, was around 5.0. LD_{50} of *P. putida* SP1 to flounder and turbot was 1.5×10^9 CFU. Biofilm developed by *P. putida* SP1 was 1- to 3-fold lower than biofilm developed by an aquatic pathogen *Pseudomonas fluorescens* TSS. The results of this study indicate that *P. putida* SP1 is a low virulence strain that can potentially be applied in the bioremediation of $HgCl_2$ contamination over a broad range of pH.

Keywords *Pseudomonas putida* · Marine environment · *mer* operon · Bioremediation of $HgCl_2$ contamination

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Introduction

Mercury is one of the most toxic heavy metals and its level of contamination in the environment has increased over a thousand fold as a consequence of anthropogenic activities, such as the discharge of wastewaters from chlor-alkali plants, the incineration of coal, and metal mining (Li et al. 2009). Even in the Arctic marine environment, the anthropogenic contribution to increasing mercury levels has been shown to be above 92% (Dietz et al. 2009). The toxicity associated with different forms of mercury has been recognized since the description of Minamata disease in Japan (Guzzi and La Porta 2008). Transformation of organic and/or inorganic mercury into less toxic metallic mercury by mercury-resistant bacteria, especially *Pseudomonas putida*, has been explored for the potential bioremediation of mercury-polluted environments (Hansen et al. 1984; Von Canstein et al. 1999; Wagner-Döbler 2003; Barkay and Wagner-Döbler 2005; Mortazavi et al. 2005; Pepi et al. 2010).

Previous studies have described the isolation of many organic and/or inorganic mercury-resistant bacteria belonging to species of the genera *Pseudomonas, Staphylococcus, Bacillus*, and *Escherichia* from various mercury-contaminated environments (Mindlin et al. 2005; Kannan and Krishnamoorthy 2006; De and Ramaiah 2007; Poulain

et al. 2007; Mirzaei et al. 2008; Bafana et al. 2010). Most mercury-resistant Gram-positive and Gram-negative bacteria possess *mer* operons as their mercury-resistant determinants, which are usually located on transposons, plasmids, or the bacterial chromosomes (Osborn et al. 1997; Nascimento and Chartone-Souza 2003). Resistance of *mer* operon contained bacterium is due to the uptake of Hg^{2+} into cytoplasm by MerT and MerP and then the reduction of Hg^{2+} to the relatively inert and less toxic Hg^0 by MerA, the mercuric reductase enzyme. Hg^0 then diffuses out of the cell through the cell membrane without the need for a dedicated transport system (Barkay et al. 2003; Nascimento and Chartone-Souza 2003).

Mercury transformation by bacteria is sensitive to important process factors; therefore, factors such as temperature, pH, ion concentrations, and carbon sources that may affect *mer*-mediated transformation systems have been previously investigated (Kholodii et al. 2000; Kholodii and Bogdanova 2002; Oehmen et al. 2009). Mortazavi et al. (2005) studied the effect of pH on the efficiency of removal of $HgCl_2$ by a *P. putida* strain. Other studies have indicated that pH is an important factor that significantly affects the concentration of bioavailable mercury, higher concentrations of mercury accumulating in the bacteria with the decreasing of pH (Golding et al. 2002; Kelly et al. 2003; Golding et al. 2008; Ahn et al. 2010). However, until now, the effect of pH on the expression of *merA* at the transcriptional level, an important process during mercury transformation, has not been examined.

In this study, we described the isolation and identification of the mercury-resistant bacterial strain SP1 from marine environment, and the cloning and localization of its *mer* operon. Our aim was to investigate the high resistance of SP1 to mercury and other heavy metals, the high efficiency in the removal of $HgCl_2$ by SP1, and the effects of pH on the expression of *merA* and on the transformation of Hg^{2+} to Hg^0. Furthermore, to determine whether SP1 was a low virulence strain, the median lethal dose (LD_{50}) and biofilm development ability of SP1 were determined. The potential of the mercury-resistant bacterial strain SP1 to be used for the removal of Hg^{2+} from marine environments was also explored.

Materials and methods

Bacterial strains, growth conditions, and chemicals

Seawater collected from Yantai coastal zone in Shandong Province, China, was concentrated 10 to 20 times by filtration through a 0.45-μm-pore-size filter membrane and plated onto 2216E media (5 g tryptone, 1 g yeast extract, 0.01 g $FePO_4$, 1 L aged seawater) supplemented with 1.2% agar. The plate was incubated at 28 °C for 2 to 3 days. *Pseudomonas fluorescens* TSS (Hu et al. 2009; Wang et al. 2009; Zhang et al. 2009), an aquaculture pathogen that can infect a number of fish species, and the isolated mercury-resistant strain were cultured at 28 °C in 2216E liquid media. *Escherichia coli* strain Top10 was cultured at 37 °C in Luria–Bertani (LB) media. Unless otherwise stated, all chemicals used in this study were purchased from Sangon (Shanghai, China). The TA cloning plasmid pMD18-T was purchased from Takara.

DNA manipulation

DNA extraction from agarose gel was performed according to the instruction of the Sangon kit. Genomic DNA from SP1 was extracted according to the method described previously (Syn and Swarup 2000). The 16S rRNA gene was PCR amplified using primers 8F and 1492R according to the method described by Lane et al. (1985). When the TA cloning strategy was employed, the purified PCR products were directly ligated into pMD18-T. Sequencing was carried out by Nuosai Sequencing Company (Beijing, China)

Detection of bacterial volatilization of $HgCl_2$

A simplified X-ray film method for detecting the production of Hg^0 by SP1 was carried out as described by Nakamura and Nakahara (1988). Briefly, SP1 and TSS were suspended in 0.07 M phosphate buffer (pH 7.0) containing 0.5 mM EDTA, 0.2 mM magnesium acetate, and 5 mM sodium thioglycollate, and incubated in a polystyrene microtiter plate with 18 μM $HgCl_2$. The plate was covered with X-ray film (Kodak) in a darkroom at 28 °C for overnight. The foggy areas on the film were the result of the reduction of the Ag^+ emulsion by mercury vapor released by the bacteria.

Metal and antibiotic tolerance and minimal inhibitory concentrations (MIC)

The metals and antibiotics used in this study are as follows: $HgCl_2$, AgCl, $CdCl_2$, $CoCl_2$, $CrCl_3$, $CuCl_2$, $PbCl_2$, $ZnSO_4$, ampicillin (Ap), kanamycin (Kn), chloramphenicol (Cm), and tetracycline (Tc). The ability of SP1 and SP1M to grow in media containing metals, except $HgCl_2$, or antibiotics was tested by adding these chemicals at the following concentrations: 0.01 mM, 0.1 mM, 0.5 mM, 1 mM, 2 mM, 5 mM, and 10 mM. For $HgCl_2$, the following concentrations were included: 50 μM, 100 μM, 200 μM, and 300 μM. The growth of bacteria was monitored after SP1 was cultured at 28 °C for 48 h. The minimal inhibitory concentrations of metals and antibiotics were defined as the

lowest concentrations that caused no visible bacterial growth (Murtaza et al. 2002).

Determination of mercury removal by SP1

SP1 was inoculated in seawater or 2216E media containing 280 μM $HgCl_2$ and cultured at 28 °C for 48 h, respectively. The supernatant and cell pellet were collected by centrifugation. The cell pellet was resuspended in lysis buffer [100 mM NaH_2PO_4, 10 mM Tris–Cl, and 8 M urea (pH 8.0)] for 1 h and then centrifuged at 13,000 rpm for 10 min to collect cell-associated mercury. The levels of both mercury remaining in the supernatant and the cell-associated mercury were determined using a cold vapor atomic absorption spectrometer (cold vapor AAS) (AFS-3000, Kchg, China). To determine the effect of pH on the removal of mercury, SP1 was inoculated into media of pH ranging from 5.0 to 9.0 and cultured at 28 °C for 48 h. The levels of both mercury remaining in the supernatant and the cell-associated mercury were determined as described above.

Cloning and localization of the *mer* operon

Three pairs of primers (merF1 and merR2, merF2 and merR3, and merCF1 and merR4) listed in Table 1 were designed according to the *mer* sequence of mercury-resistant *Pseudomonas*/*Xanthomonas* strains isolated from a mercury mine in Kirgizia, Central Asia, submitted to the National Center for Biotechnology Information (NCBI) under the accession number X98999 reported by Kholodii et al. (1997). PCR products were separated, purified, and then ligated into pMD18-T. *E. coli* transformants were selected and sequencing of the DNA fragment was carried out. To determine the localization of the *mer* operon, plasmid curing and PCR analysis were carried out. Plasmid harbored in SP1 was knocked out according the method described by De et al. (2003) to generate strain SP1M. Plasmids were isolated from both SP1 and SP1M using the method of Kado and Liu (1981). SP1M was verified to be a derivative of SP1 by 16S rRNA analysis. PCR reactions using the genomic DNA from SP1, SP1M, and the extracted plasmid as template were carried out using primers merAF1 and merAR1, respectively (Table 1).

Real-time PCR (RT-PCR) to analyze the expression of *merA*

To determine whether 16S rRNA was an appropriate internal control, northern dot blotting was carried out as described by Zhang et al. (2008) with minor modifications. RT-PCR was carried out as described by Zhang (2008). RNA was extracted from cells using the total RNA isolation system (Invitrogen). RT-PCR was carried out in an ABI 7500 real-time detection system (Applied Biosystems) by using the SYBR *Premix Ex Taq* RT-PCR kit (Takara). The primers used for RT-PCR of the *merA* gene were merRTF1 and merRTR1 (Table 1). 16S rRNA was amplified with primers 933F and 16SRTR1 (Table 1). Dissociation analysis of the amplification products was performed at the end of each PCR run to confirm that only one PCR product was amplified and detected. The comparative threshold cycle method ($2^{-\Delta\Delta CT}$ method) was used to analyze the relative mRNA expression.

Determination of LD_{50} by experimental infection

To determine whether the retention of SP1 in the environment was pathogenic, LD_{50} of SP1 was determined using flounder and turbot as animal models. LD_{50} was determined as described by Wang et al. (2009). Briefly, SP1 was

Table 1 Primers used in this study

Primer	Sequence (5′→3′)	Reference
merF1	TAAGACTGCTGCTGGTAGTGGGTG	Kholodii et al. (1997)
merR2	GCGAGCCGCTTAGGGATTGTAT	Kholodii et al. (1997)
merF2	TGCTGCCTCTGTTTGCTGGACT	Kholodii et al. (1997)
merR3	GCCCGATACCGGACTCAAAGG	Kholodii et al. (1997)
merCF1	CATATGGCAAATCCTCTCAATC	This study
merR4	CCCGATGCACGCCCGGAATCTTCT	This study
merRTF1	TACCGCCGTCTGGATCAGTTCTCCT	This study
merRTR1	CGAAACCGACAGCCGCACCTT	This study
merAF1	CATATGACCGAACTGAAAATCATTGGTAT	This study
merAR1	CTCGAGCCCTGCGCAGCAGG	This study
933F	GCACAAGCGGTGGAGCATGTGG	Zhang et al. (2008)
16SRTR1	CGTGTGTAGCCCTGGTCGTA	Zhang et al. (2008)
8F	AGAGTTTGATCCTGGCTCAG	Zhang and Sun (2007)
1492R	GGTTACCTTGTTACGACTT	Zhang and Sun (2007)

cultured to an OD_{600} of 1.0 and suspended in sterilized phosphate-buffered saline (PBS). Healthy flounder and turbot (approximately 11 g) were reared at 20 to 22 °C in seawater and fed daily with commercial dry pellets. Thirty flounder or 30 turbot were divided randomly into five groups (six fish per group) respectively and injected intraperitoneally (i.p.) with 100 μL of SP1 suspension of concentrations ranging from 5.0×10^8 to 5.0×10^{10} CFU mL^{-1}. Mortality was monitored over a period of 14 days after the challenge and the LD_{50} was determined using the Probit analysis tool of the SPSS 15.0 software (SPSS Inc., USA).

Biofilm development analysis

The ability of TSS and SP1 to form biofilms on a polystyrene microtiter dish (Costar, USA) was determined as described by Xu et al. (2006). Briefly, TSS and SP1 were cultured in a 96-well polystyrene microtiter plate at 28 °C for 12 h and 24 h, respectively. Unattached cells were washed away five times with PBS. The attached cells were treated with Bouin's fixative for 1 h and stained with 1% crystal violet solution for 30 min. Plates were then rinsed with running water and bound crystal violet was dissolved in ethanol for 30 min. The absorbance at 570 nm was measured with an ultraviolet and visible spectrophotometer (Beckman, USA).

Database search and nucleotide sequence accession numbers for 16S rRNA and the *mer* operon

Searches for nucleotide and amino acid sequence similarities were conducted using the BLAST programs from the NCBI. The nucleotide sequences of the 16S rRNA genes and the *mer* operon of SP1 have been deposited in the GenBank database under the accession numbers HM217131 and HM217134. SP1 isolate was deposited with the China General Microbiological Culture Collection (CGMCC, Beijing, China, accession number CGMCC no. 3887).

Results

Screening, isolation, and genetic identification of mercury-resistant bacterium

Collected seawater was plated onto 2216E solid media supplemented with 18 μM $HgCl_2$. After incubation at 28 °C for 2 to 3 days, one colony resistant to $HgCl_2$ appeared and was named SP1. To genetically verify the identity of SP1 and further position the isolate within genus or species, the 16S rRNA genes of SP1 were amplified by PCR and the PCR products were purified and submitted directly for sequencing. Comparison with the known 16S rRNA gene sequence data indicated that the 100% matches for the16S rRNA gene sequence of SP1 were those of *P. putida* DSM 1819, type strain of *P. putida*, *P. putida* KT-ql-116 and WXZ-19, and *Pseudomonas* sp. strains JW60.1a and VS05_36, whose accession number were Z76667, FJ611926, EF440613, FN556575, and FJ662897, respectively. These results indicated that SP1 is a member of *P. putida*.

Cloning and localization of the *mer* operon of *P. putida* SP1

A 2,817-bp fragment amplified using merF1/merR2 as primers, a 2,601-bp fragment amplified using merF2/merR3 as primers, and a 561-bp fragment amplified using merCF1/merR4 as primers are shown in Fig. 1a. The amplified DNA fragment was sequenced and assembled. Analysis of the sequenced nucleotide demonstrated that it shared 99% identity with the *mer* operon located on Tn5041. The cloned DNA fragment from *P. putida* SP1 contained *merR* and the *mer* operon including the integral *merT*, *merP* and *merA* genes, the accessory *merC* gene, and the function unknown *orfY* gene. The localization of *mer* operon was shown to be on the chromosome of *P. putida* SP1, as PCR amplification of *merA* gene with primers merAF1 and merAR1, using the chromosomal DNA from plasmid missing strain SP1M as a template, showed a positive signal and PCR amplification of *merA* gene, using the plasmid extracted from *P. putida* SP1 as a template, showed a negative signal (Fig. 1b). There was no reduction in metal and antibiotic resistance in strain *P. putida* SP1M compared with *P. putida* SP1, suggesting that multiple resistance determinants were also located on the bacterial chromosome (Table 2).

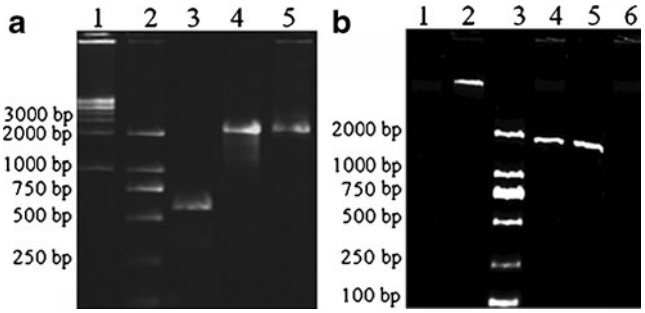

Fig. 1 Cloning of *mer* operon of *P. putida* SP1 (**a**) and detection of plasmids and the *merA* gene of *P. putida* SP1 and SP1M (**b**). **a** DNA marker (*lane 1*), DNA marker (*lane 2*), DNA fragment amplified with primers merCF1 and merR4 (*lane 3*), DNA fragment amplified with primers merF2 and merR3 (*lane 4*), DNA fragment amplified with primers merF1 and merR2 (*lane 5*). **b** Plasmids were extracted from *P. putida* SP1M (*lane 1*) and SP1 (*lane 2*), DNA marker (*lane 3*), the *merA* gene was amplified with primers merAF1 and merAR1 using genomic DNA of *P. putida* SP1M (*lane 4*), genomic DNA of SP1 (*lane 5*), and plasmids (*lane 6*) as template, respectively

Table 2 Tolerance of SP1 and SP1M to metals and antibiotics[a]

Metal/antibiotics	MIC (mM)
$HgCl_2$	0.3
AgCl	0.01
$CdCl_2$	1
$CoCl_2$	1
$CrCl_3$	8
$CuCl_2$	2
$PbCl_2$	1
$ZnSO_4$	1
Ampicillin	0.6
Kanamycin	0.62
Chloramphenicol	0.6
Tetracycline	0.1

[a] The MIC for SP1M is the same compared with that for SP1 shown in the table

Growth characteristics of *P. putida* SP1 and SP1M

To characterize the growth of bacteria in media containing $HgCl_2$, strains were cultured at 28 °C with shaking (120 rpm), either with 18 μM $HgCl_2$ added to the media at an OD_{600} of 0.2 or with no $HgCl_2$ added as a control. The growth patterns of *P. putida* SP1 and SP1M in the media containing $HgCl_2$ are shown in Fig. 2. When $HgCl_2$ was added to the media at an OD_{600} of 0.2, there was a prolonged lag phase in the growth of *P. putida* SP1. The growth of *P. putida* SP1 then entered an exponential phase and reached the same maximum cell density as *P. putida* SP1 grown in media without $HgCl_2$. However, growth of another *Pseudomonas* strain TSS, which is referred as a mercury-sensitive bacterial strain, was completely inhibited by the addition of 18 μM $HgCl_2$. The growth of *P. putida* SP1 and SP1M was the same in the media that did not contain $HgCl_2$. In the presence of $HgCl_2$, growth of *P. putida* SP1M showed the same trend as *P. putida* SP1. It also possessed a long lag phase and then entered an exponential growth phase. Unexpectedly, in the late lag phase and throughout the exponential growth phase, the growth of *P. putida* SP1M was slightly better than that of *P. putida* SP1.

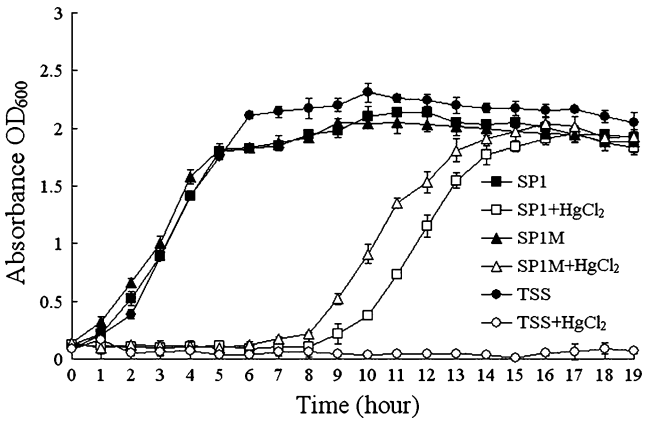

Fig. 2 Growth of mercury-resistant bacteria (*P. putida* SP1 and SP1M) and mercury-sensitive strain (*P. fluorescens* TSS) in 2216E media. $HgCl_2$ was added at OD_{600} of 0.2 to the final concentration of 18 μM. *Symbols* indicate growth of *P. putida* SP1 in the absence of $HgCl_2$ (*filled squares*), in the presence of $HgCl_2$ (*open squares*), growth of *P. putida* SP1M in the absence of $HgCl_2$ (*filled triangles*), in the presence of $HgCl_2$ (*open triangles*), and growth of *P. fluorescens* TSS in the absence of $HgCl_2$ (*filled circles*), in the presence of $HgCl_2$ (*open circles*). Aliquots were taken at different time points for measurements of absorbance at 600 nm. Data are the means for at least three independent experiments and are presented as the means±SE

Metal and antibiotic tolerance of *P. putida* SP1 and SP1M

The growth of *P. putida* SP1 in the media amended with different concentrations of $HgCl_2$ is shown in Fig. 3. *P. putida* SP1 grew more slowly with the increasing concentration of $HgCl_2$ amended into the media. After being cultured for 48 h, *P. putida* SP1 obviously showed growth in the media amended with 200 μM $HgCl_2$, but showed slight growth in the media amended with 300 μM $HgCl_2$. Therefore, the MIC of $HgCl_2$ for *P. putida* SP1 was found to be 300 μM, suggesting that *P. putida* SP1 was highly resistant to mercury. Both *P. putida* SP1 and SP1M were tested for metal and antibiotic tolerance. The MICs of AgCl, $CdCl_2$, $CoCl_2$, $CrCl_3$, $CuCl_2$, $PbCl_2$, $ZnSO_4$, and antibiotics Ap, Kn, Cm, and Tc for *P. putida* SP1 and SP1M are listed in Table 2. These results indicated that both *P. putida* SP1 and SP1M are able to grow in 2216E media containing high concentrations of $CdCl_2$, $CoCl_2$, $CrCl_3$, $CuCl_2$, $PbCl_2$, and $ZnSO_4$, respectively, and that *P. putida* SP1M did not demonstrate reduced resistance to any of these metals or antibiotics compared with *P. putida* SP1.

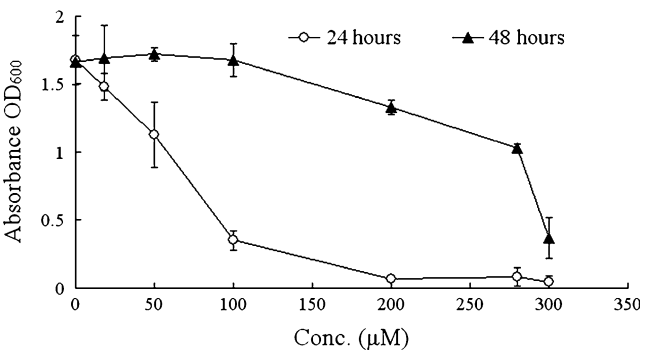

Fig. 3 Effect of concentration of $HgCl_2$ on the growth of *P. putida* SP1. *P. putida* SP1 was grown in 2216E media amended with different concentrations of $HgCl_2$. Aliquots were taken at different time points for the measurement of absorbance at 600 nm. Data are the means for at least three independent experiments and are presented as the means±SE

Removal of $HgCl_2$ by *P. putida* SP1

To determine the highest concentration of $HgCl_2$ that *P. putida* SP1 was able to tolerate, several $HgCl_2$ concentrations between 200 μM and 300 μM were amended into the media. The result demonstrated that *P. putida* SP1 grew well in 2216E media containing 280 μM $HgCl_2$ (Fig. 3). *P. putida* SP1 was then inoculated into pure seawater containing 280 μM $HgCl_2$ and incubated for 48 h. The remaining concentration of $HgCl_2$ in the seawater was then measured by cold vapor AAS. These results showed that 89% of the total mercury was removed by *P. putida* SP1. To investigate the survival of *P. putida* SP1 in seawater, 1.0×10^8 CFU mL^{-1} of *P. putida* SP1 was inoculated into seawater containing 280 μM $HgCl_2$, and aliquots were taken at various time points for OD_{600} measurements and plate counts. The results showed that the number of viable bacteria was highest in the early days due to the remaining nutrient introduced into the seawater and declined over time (Fig. 4). The simplified X-ray film method was used to determine whether Hg^0 was formed. *P. putida* SP1 clearly induced a volatilization reaction in the buffer containing $HgCl_2$; however, no reaction was observed in the control wells or in wells containing *P. fluorescens* TSS, the mercury-sensitive strain (Fig. 5).

To improve the efficiency of mercury removal by *P. putida* SP1, this strain was inoculated into fresh 2216E media including 280 μM $HgCl_2$. After shaking at 28 °C for 48 h, both the mercury remaining in the supernatant and cell-associated mercury were subjected to cold vapor AAS to determine the mercury level. The remaining mercury concentration in the supernatant was 0.23 μM, and the mercury concentration in the cell pellet was 0.383 μM. These results indicated that by adding nutrients to seawater, a comprehensive increase in efficiency was achieved, with almost 100% of total mercury removed from the aquatic environment. It was hypothesized that the Hg^{2+} removed by

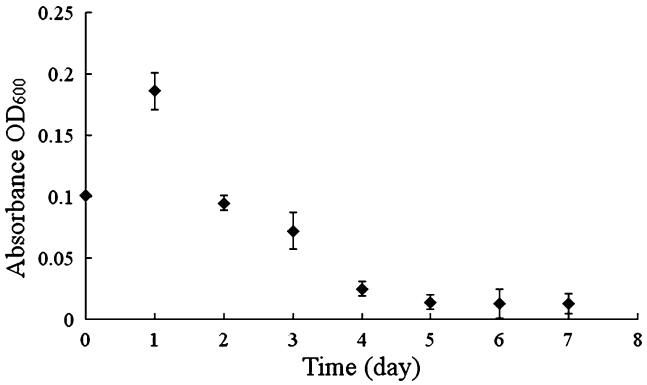

Fig. 4 Survival of *P. putida* SP1 in seawater containing 280 μM $HgCl_2$. Aliquots were taken at different time points post-inoculation and measured the absorbance at 600 nm. Data are the means for at least three independent assays and are presented as the means±SE

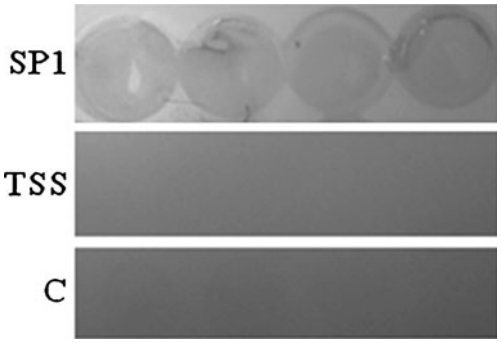

Fig. 5 X-ray film capture of volatilized $HgCl_2$. *P. putida* SP1 and *P. fluorescens* TSS were inoculated into a 96-well polystyrene microtiter plate containing media amended with $HgCl_2$. The plate was then covered with X-ray film (Kodak) in the darkroom at 28 °C for overnight

P. putida SP1 was transformed into Hg^0 by the *mer* operon of *P. putida* SP1 and then Hg^0 diffused out of the culture.

Effects of pH on the growth of *P. putida* SP1 and the removal of $HgCl_2$ by *P. putida* SP1

To determine the effect of pH on the growth of *P. putida* SP1, this strain was inoculated into media of pH ranging from 4.0 to 10.0 and grown at 28 °C for 48 h. As shown in Table 3, the growth of *P. putida* SP1 showed a wide range of permissive pH between 5.0 and 9.0. The optimal pH for the growth of *P. putida* SP1 in media without $HgCl_2$ was 6.0–7.0; however, *P. putida* SP1 grew best in weakly alkali media of pH 8.0–8.5 in the presence of 18 μM $HgCl_2$. Table 3 shows the final pH of culture of *P. putida* SP1 grown for 48 h in media of different pH with or without $HgCl_2$. Growth of *P. putida* SP1 affected the pH of the media. After *P. putida* SP1 was cultured for 48 h, the final pH of the culture moved to alkaline environment and increased 0.14–1.55 units, respectively. The media of acid pH varied with the wide range and the media of alkaline pH varied with the small range.

To determine the effect of pH on the removal of $HgCl_2$ by *P. putida* SP1, media of pH ranging from 5.0 to 9.0 was used. The mercury removed at each pH point was expressed as the percentage of the mercury that was initially added into the media. As shown in Table 3, the results indicated that alkaline condition, pH ranging from 8.0 to 9.22, was more suitable for *P. putida* SP1 to remove $HgCl_2$. The removal efficiency of *P. putida* SP1 at initial pH 8.0 and 9.0, whose corresponding final pH was 8.40 and 9.22, respectively, was significantly higher than the removal efficiency at initial pH 7.0, whose corresponding final pH was 7.68. The difference in removal efficiency at acid and neutral conditions was insignificant. Therefore, the result indicated that alkaline condition could facilitate *P. putida* SP1 to remove $HgCl_2$, and acidic and neutral condition led

Table 3 Effects of pH on the growth of *P. putida* SP1 and removal of Hg^{2+} by SP1

Initial pH	Final pH		Relative absorbance OD_{600} (%)[a]		Percentage of Hg^{2+} removed (%)[b]
	− Hg^{2+}	+ Hg^{2+}	− Hg^{2+}	+ Hg^{2+}	
3.93±0.01	–	–	–	–	–
4.93±0.02	6.42±0.04	5.45±0.16	100.00	87.97±4.82	79.78±1.55
5.94±0.06	7.06±0.04	6.71±0.02	109.01±9.81	87.43±1.55	83.33±6.82
7.05±0.04	7.80±0.02	7.68±0.02	99.72±7.53	87.43±6.21	81.33±5.36
8.06±0.03	8.47±0.16	8.40±0.09	95.41±6.24	94.42±5.67	99.57±5.04
8.97±0.05	9.25±0.17	9.22±0.06	85.67±8.61	85.09±3.82	96.86±4.38
9.99±0.04	–	–	–	–	–

[a] Relative absorbance OD_{600} is expressed as percentage of the absorbance of culture of *P. putida* SP1 grown at pH 5.0 in the absence of $HgCl_2$

[b] Percentage of Hg^{2+} removed by *P. putida* SP1 is expressed as the percentage of initial concentration of Hg^{2+} that was added into the media of different pH

to reduction in the mercury removal efficiency of *P. putida* SP1.

Effect of pH on the expression of *merA*

To investigate the effect of pH on the expression of *merA*, a culture of *P. putida* SP1 (OD_{600} of approximately 0.5) was divided into five parts and centrifuged at 10,000 rpm for 5 min to collect the bacteria. The bacteria were then added into media of different pH ranging from 5.0 to 9.0. After being cultured for another 15 min, the growth of *P. putida* SP1 in the media of different pH was not significantly different ($P>0.05$) and no change of pH was observed. Total RNA was extracted from cells harvested at different growth conditions and used for both northern blot analysis of 16S rRNA transcription and RT-PCR analysis of *merA* mRNA expression. The result of northern dot blotting using equal amounts of RNA showed that differences in the transcription levels of 16S rRNA were between 0.9- and 1.5-fold among the *P. putida* SP1 strains grown at different pH, suggesting that pH scarcely affected the transcription of 16S rRNA of *P. putida* SP1. Hence, 16S rRNA was used as an internal control in the RT-PCR analysis of *merA*. As shown in Fig. 6, expression of *merA* was significantly influenced by pH fluctuations and the mRNA level increased with the decrease of pH. The expression of *merA* was strongly stimulated particularly when the pH of the media was lower than 7.0.

LD_{50} and biofilm development analysis

To estimate whether *P. putida* SP1 was a low virulence or nonpathogenic strain used for bioremediation, the LD_{50} of *P. putida* SP1 was determined and biofilm development assays were carried out. Both flounder and turbot were challenged with 100 μL of different concentrations of *P. putida* SP1 suspension and were monitored for mortality. Although different injection doses exhibited varied death number, both flounder and turbot began to die at the fourth day and the death lasted to the seventh day when the fish was challenged with 5.0×10^9 CFU *P. putida* SP1 (Fig. 7). No death was observed when the two kinds of fish were challenged with 5.0×10^7 CFU *P. putida* SP1. The survival graph indicated that both flounder and turbot showed prompt death after being challenged with *P. putida* SP1. LD_{50} of *P. putida* SP1 was calculated using the Probit analysis tool of the SPSS 15.0 software. LD_{50} of *P. putida* SP1 to flounder or turbot was 1.5×10^9 CFU, which was a relatively high dose. We also compared the ability of *P.*

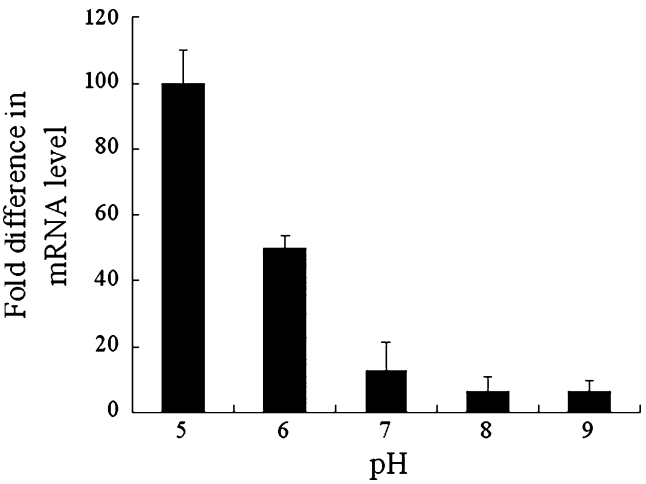

Fig. 6 Effect of pH on the expression of *merA*. *P. putida* SP1 was propagated at 28 °C to an OD_{600} of 0.5 and cells were collected by centrifugation. Cells were resuspended in media of pH ranging from 5.0 to 9.0 with 18 μM $HgCl_2$ and cultured at 28 °C for another 15 min. Total RNA was extracted from cells and used for RT-PCR. The mRNA level of *merA* was normalized to that of 16S rRNA. Data are the means for three independent experiments and are presented as the means±SE

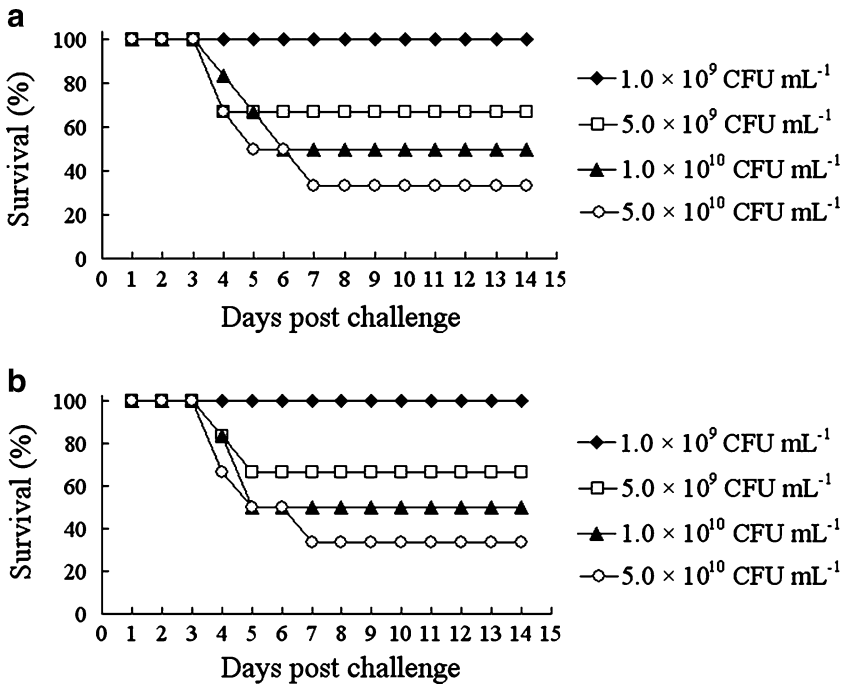

Fig. 7 Survival curve of flounder (**a**) and turbot (**b**) after the fish were challenged with different concentrations of *P. putida* SP1 suspension. Flounder and turbot were i.p. injected with 100 μL of *P. putida* SP1 suspension of 5.0×10^8 CFU mL^{-1}, 1.0×10^9 CFU mL^{-1}, 5.0×10^9 CFU mL^{-1}, 1.0×10^{10} CFU mL^{-1}, and 5.0×10^{10} CFU mL^{-1}. Mortality was monitored over a period of 14 days after the challenge

putida SP1 to form biofilm with that of *P. fluorescens* TSS. The result demonstrated that although *P. putida* SP1 could develop biofilm, the biofilm production of *P. putida* SP1 was 1- to 3-fold less compared with the biofilm produced by *P. fluorescens* TSS. Prolonged incubation time did not result in increases in the biofilms developed by *P. putida* SP1, unlike the increasing biofilm production pattern of *P. fluorescens* TSS.

Discussion

Many mercury-resistant bacteria have been isolated from various environments and have been shown to have promising applications in mercury removal in both laboratory and pilot plant scales (Von Canstein et al. 1999; Wagner-Döbler 2003; Barkay and Wagner-Döbler 2005; Mortazavi et al. 2005; Pepi et al. 2010). Bacterial strains with potential to be used for bioremediation were thought to undergo selection pressures in the presence of antibiotics, heavy metals, and organic solvents (Hideomi et al. 1977). In this study, the bacterial strain *P. putida* SP1 was able to survive in 2216E media amended with 280 μM $HgCl_2$. *P. putida* SP1 also exhibited higher levels of resistance to antibiotics and a variety of toxic heavy metals than did the strain *Pseudomonas* sp., *Proteus* sp., *Aeromonas* sp., *Enterobacteriaceae* sp., and *Xanthomonas* sp. described previously (De et al. 2003; Bafana et al. 2010). To our knowledge, this was the highest concentration of $HgCl_2$ that the *Pseudomonas* sp. strains could tolerate. Compared with the *P. putida* Spi3 isolated from river sediments, *P. putida* SP1 could remove mercury in higher efficiency at higher NaCl concentration. NaCl at the concentration of less than 24 g L^{-1} was suitable for *P. putida* Spi3 to remove mercury; however, *P. putida* SP1 could remove almost 100% of Hg^{2+} from seawater, in which the concentration of NaCl is up to about 36 g L^{-1} (Von Canstein et al. 1999). *P. putida* SP1 possessed a *mer* operon as a mercury-resistant determinant like most of the other mercury-resistant bacteria, and the *mer* operon of *P. putida* SP1 was located on the chromosomal DNA as described in other strains (De et al. 2003; Bafana et al. 2010). Because multiple resistance genes are often located on mobile genetic elements (Mindlin et al. 2001; Partridge et al. 2001; Barkay et al. 2003) and genes encoding for heavy metal resistance are often linked to antibiotic resistance genes on the same mobile element (Mindlin et al. 2002; Barkay et al. 2003), the similarity between the *mer* operon of *P. putida* SP1 and the *mer* operon located on Tn5041 suggested that the environmental bacterium *P. putida* SP1 may have acquired this chromosomal *mer* operon and other heavy metal or antibiotic resistance determinants through transposable elements that confer resistance to $HgCl_2$ and a variety of other xenobiotics.

As an important environmental factor, pH has been previously studied about its effect on bacterial reduction of Hg^{2+} (Mortazavi et al. 2005). In this study, we found that alkaline conditions were optimal condition for *P. putida* SP1 to volatilize Hg^{2+}, which was consistent with the fact that *P. putida* SP1 was a strain isolated from marine environment. Expression of *merA* was facilitated under acidic conditions, such as pH 5.0. This finding was consistent with the previous studies that more Hg^{2+}, the necessary factor to

induce the expression of *merA*, accumulated in the cell with the decrease of pH (Kelly et al. 2003; Ahn et al. 2010). Considering all of these results, it was suggested that, besides the expression of *merA* and the concentration of Hg^{2+} accumulated in bacteria, the transformation of Hg^{2+} by *P. putida* SP1 may also require other specific detoxification reactions to contribute to its ability to volatilize Hg^{2+}, such as the production of DNDPH by bacteria.

Mercury-resistant bacteria are an important alternative tool for bioremediation because of their simplicity, lack of secondary contamination, and low cost compared with other treatment technologies (Singh et al. 2008). Although *P. putida* SP1 was present at barely detectable level in oligotrophic marine niche, it still volatilized 89% of total mercury. The higher efficiency that was achieved by the addition of nutrients into seawater was partially due to the gradually increasing population of *P. putida* SP1 in the environment. As several isolates of *P. putida* were recently reported to be of pathogenic significance (Bouallèguea et al. 2004; Altinok et al. 2006), we estimated the pathogenicity of *P. putida* SP1 to secure its use for bioremediation. The LD_{50} of *P. putida* SP1 to two kinds of important marine fish was 1,000-fold higher than the LD_{50} of *P. fluorescens* TSS ($10^{6.1}$) (Wang et al. 2009), demonstrating that *P. putida* SP1 was drastically attenuated in its overall bacterial virulence and potentially was a bacterial strain with low pathogenicity. Furthermore, as biofilm is one of the most important parameters associated with pathogenesis, the reduced biofilm development by *P. putida* SP1 compared with *P. fluorescens* TSS, whose biofilm had been shown to be involved in pathogenesis, further strengthened the notion that *P. putida* SP1 was a low virulence strain that could be used in the bioremediation in mercury-polluted marine environments (Parsek and Singh 2003; Hu et al. 2009).

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