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ArsM-mediated arsenite volatilization is limited by efflux catalyzed by As efflux transporters

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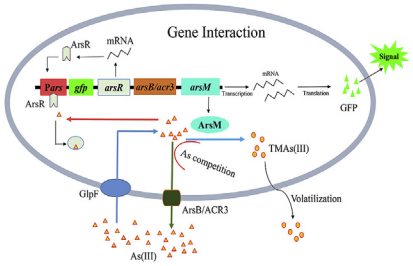
HIGHLIGHTS

- A novel As biosensor for intracellular As content measurement was developed.
- The different As efflux transporters exhibited a significant difference in As efflux activity.
- As efflux transporters were closely related to limited As volatilization and As methylation rate.

GRAPHICAL ABSTRACT



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ABSTRACT

Arsenic (As) methylation is regarded as an efficient strategy for As contamination remediation by As volatilization. However, most microorganisms display low As volatilization efficiency, which is possibly linked to As efflux transporters competing for cytoplasmic As(III) as a substrate. Here, we developed two types of As biosensors in *Escherichia coli* to compare the As efflux rate of three efflux transporters and to further investigate the correlation between As efflux rates and As volatilization. The engineered As-sensitive *E. coli* AW3110 expressing $arsB_{RP}$, $acr3_{RP}$ or $arsB_{EC}$ displayed a higher As resistance compared to the control. The fluorescence intensity was in a linear correlation in the range of 0–2.0 μmol/L of As(III). The intracellular As(III) concentration was negatively related to As efflux activity of As efflux transporter, which was consistent with the As resistance assays. Moreover, *arsM* derived from *R. palustris* CGA009 was subsequently introduced to construct an *E. coli* AW3110 co-expressing *arsB*/*acr3* and *arsM*, which exhibited higher As(III) resistance, lower fluorescence intensity and intracellular As concentration compared to the engineered *E. coli* AW3110 expressing only *arsB*/*acr3*. The As volatilization efficiency was negatively related to As efflux activity of efflux transporters, the recombinants without *arsB*/*acr3* displayed the highest rate of As volatilization. This study provided new insights into parameters affecting As volatilization with As efflux being the main limiting factor for As methylation and subsequent volatilization in many microorganisms.

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1. Introduction

Arsenic (As) is a toxin ubiquitously present in the environment (Mandal and Suzuki, 2002). Almost all microorganisms have acquired different pathways to reduce the toxicity of arsenic compounds, such as As(V) (arsenate) reduction, As(III) (arsenite) oxidation, As efflux and methylation as well as As demethylation (Ben Fekih et al., 2018; Kruger et al., 2013; Yan et al., 2018; Zhu et al., 2017). These As transformations not only play an important role in driving the As bio-geological cycle, but also inspired people to employ these mechanisms to solve serious environmental problems. Previous studies on *Rhodopseudomonas palustris* and *Methanosarcina acetivorans* C2A had found that As methylation converted highly toxic As(III) into less toxic volatile TMAs(III) (trimethyl-arsine) (Qin et al., 2006; Wang et al., 2014b). Thus, As methylation can be used as an effective strategy for remediating As pollution. However, with the exception of a few bacteria with a high As volatilization rate, such as *Arsenicibacter rosenii* SM-1 (Huang et al., 2016) and possibly *Prochlorococcus* (Giovannoni et al., 2019; Saunders and Rocap, 2016), most microorganisms exhibited a low As volatilization rate that is dependent on As methylation (Wang et al., 2014a). Studies have shown that both As methylation and volatilization were affected by environmental factors such as pH (Zhao et al., 2013), temperature (Huang et al., 2015), organic material (Huang et al., 2012; Yang et al., 2018) and moisture content (Shimizu et al., 2011). Environmental factors might alter the expression level and activity of gene products of microbial *arsMs*, which are the genes encoding As(III) S-adenosylmethionine methyltransferase (ArsM) responsible for As methylation, thereby affecting the As methylation rate (Zhao et al., 2013). Although changes in external conditions could be used to explain the reason for the low efficiency of As methylation in many microorganisms as one possibility, there have been few studies on internal factors limiting microbial As methylation. At present, studies have shown that As demethylation converted MAs(III) (monomethylarsine) to inorganic As thereby limiting As methylation efficiency (Su et al., 2015; Yan et al., 2017). It is inferred that processes competing with As methylation catalyzed by ArsM for the substrate As(III), would limit the As methylation efficiency in microorganisms.

R. palustris is a model organism with extraordinary metabolic versatility for studying anaerobic degradation of aromatic compounds, hydrogen production, nitrogen fixation, photosynthesis and As metabolism (Liu et al., 2015; Wu et al., 2019; Zhao et al., 2011). *R. palustris* has extensively been applied for wastewater treatment, bioremediation, hydrogen production and electricity generation (Fu et al., 2014; Liu et al., 2015; Zhao et al., 2011). Qin et al. (2006) first identified and characterized ArsM from *R. palustris* CGA009, providing a better understanding of this bacterial As methylation pathway at a molecular level. Since then, a series of studies have been conducted with $ArsM_{RP}$, which exhibited a high methylation activity and was considered a suitable candidate for application in processes involving As methylation (Chen et al., 2014a; Ke et al., 2019; Liu et al., 2011; Yuan et al., 2008). However, Zhao et al. (2015) showed that $ArsM_{RP}$ conferred a relatively limited As volatilization capacity to *R. palustris* strain CGA009, but interestingly, $ArsM_{RP}$ heterologously expressed in As-sensitive *Escherichia coli* AW3110 showed a significantly higher As volatilization rate (Zhu et al., 2008). Previous studies have shown that *Arsenicibacter rosenii* SM-1 exhibited a higher As volatilization ($47.6 \pm 18.4\%$) due to the absence of genes encoding As efflux transporters on its genome (Huang et al., 2016), and engineered *E. coli* strains lacking a gene encoding an As efflux transporter displayed a high As volatilization rate (~ 10.39%) (Ke et al., 2019). In addition, studies have shown that deletion or inactivation of genes encoding As efflux transporters increased intracellular As accumulation (Sousa et al., 2015). Based on previous studies examining the transcriptional level of *arsM* in *R. palustris* CGA009 (Zhao et al., 2015) and the postulated As methylation mechanism (Bentley and Chasteen, 2002; Dheeman et al., 2014; Hayakawa et al., 2005), we assumed that the presence of As efflux transporters in the cell would reduce the intracellular As concentration. Since As(III) is the substrate for As methylation, lowering the intracellular As(III) content would limit As methylation and subsequent volatilization.

Arsenic efflux is the most important As detoxification mechanisms of microorganisms (Garbinski et al., 2019). To date, six types of As efflux transporters (ArsB, Acr3, ArsJ, ArsP, MSF1, and ArsK) have been found in various As-resistant bacteria, and each translocates different types of As compounds (Garbinski et al., 2019). Regarding As efflux transporters, the reported studies focused on the function or the selectivity of metalloids by mutation or silence or heterologous expression of these proteins (Shi et al., 2018; Sousa et al., 2015). However, little information is available about As efflux transporters limiting the efficiency of microbial As methylation.

Arsenic is ubiquitous in nature and poses a serious threat to human health and the environment. Therefore, it is of great significance for food safety and human health to detect the As content in the environment qualitatively or quantitatively. Currently, traditional methods for As detection including ion-pairing reversed phase chromatography with inductively coupled plasma mass spectrometry and ion chromatography hydride-generation atomic fluorescence spectrometry, while with high sensitive to low As concentrations, these analytical methods are disadvantaged by their reliance on expensive equipment and lengthy time delays due to the collection, transport and processing of samples (Kaur et al., 2015). Therefore, it is particularly important to develop a method that is simple, fast, low cost, low detection limit and high sensitivity. Conventional methods to examine As efflux is by using a radioactive substrate (Drobna et al., 2005) or by using expensive instrumentation such as high performance liquid chromatography (HPLC) coupled with hydride generation-atomic absorption spectroscopy (HG-AAS) (Styblo et al., 1995), electrospray ionization tandem mass spectrometry detection (HPLC-ESI-MS/MS) or inductively coupled plasma mass spectroscopy (ICP-MS) (McKnight-Whitford et al., 2010). More recently, microbial As biosensors, a novel complementary detection method, was frequently used in As detection with great potential in high-throughput real-time. Also, this approach does not require tedious sample preparation, complex pretreatment procedures, expensive instruments and professional personnel.

Arsenic biosensors was constructed according to the structure of *ars* operon, including regulatory zones, manipulation zones, and reporter genes for signal detecting (Kaur et al., 2015). The mechanism of microbial As biosensors is based on the interaction between an *ars* regulator (ArsR) and manipulation zones. ArsR is bound to promoter in manipulation zones. Once the complex binds to targeted As species, the affinity of ArsR to promoter is greatly reduced and ArsR disassociates from the promoter, activating gene expression by derepression (Arruda et al., 2016). Based upon the mechanism, the signal intensity generated by the protein encoded by the reporter gene is positively correlated with bioavailable As concentration. Common reporter genes used in biosensors include *lacZ*, *luc*, *lux*, *yfp* and *gfp* (Fujimoto et al., 2006; Hu et al., 2010; Roberto

et al., 2002; Stocker et al., 2003). Microbial As biosensors detect As based on the interaction between As(III) and a As(III)-responsive regulator coupled with a reporting signal such as fluorescence (Roberto et al., 2002) or LacZ (Scott et al., 1997), therefore the As(III) concentration is positively correlated to signal intensity. Scott et al. (1997) constructed an As biosensor to monitor As in the environment using β-galactosidase as the reporter with 7.7 μg/μL detection limit but the whole procedure consumed 7 h. The other research later improved the performance of As biosensors. In 2002, Roberto et al. (2002) constructed an As biosensor to detect environmental traces of As, improving the detection limit to 1 μg/L using GFP as reporter. Siddiki et al. (2011) constructed a biosensor with a sensitivity of 5 g/L that needed only 30 min for whole test. Later, the biosensor was improved to have a better sensitivity of 1 g/L (Siddiki et al., 2012). Chen et al. (2014b) developed a biosensor that could specifically detect organic As in herbicides and growth promoters with a sensitivity of 15.4 g/L. Although the prospects are promising, microbial biosensors are not widely used due to variations in media composition, culture time and other limiting factors, compared to the traditional method of determining As in the environment. However, based on the mechanism of microbial biosensors mentioned above, we can theoretically establish a biosensor-based method to study how the actions of certain gene products influence the concentration of substrates and subsequently induction of other genes regulated by these substrates.

To test mentioned hypothesis above, herein, we constructed two types of As biosensors to investigate the rate of As efflux by ArsB/ACR3 and determine the effect of As(III) efflux on microbial As volatilization, respectively. The genetic elements of the biosensor consisted of *arsR* encoding a transcriptional regulator and its cognate operator/promoter region, upstream of a promoterless *gfp* gene, which allowed for a detectable fluorescence output regulated by ArsR, in response to As. This work established an As biosensor for monitoring intracellular As concentration to compare the As efflux activities of three As efflux transporters and for the first time examined the correlation between As efflux transporters and As volatilization catalyzed by ArsM (shown as Fig. S1), and provided an explanation for the low methylation efficiency of microorganisms and an effective strategy for screening microorganisms with a higher As volatilization rate.

2. Material and methods

2.1. Strains, plasmids and reagents

Rhodopseudomonas palustris CGA009 (ATCC BAA-98) was obtained from the American Type Culture Collection (ATCC, USA). As-sensitive strain *E. coli* AW3110(DE3)(Δ*arsRBC*) was a gift from Professor Yongguan Zhu of the Institute of Urban Environment, Chinese Academy of Science. pUC19-P*ars-gfp-arsRC* was synthetized by Nanjing Kingsley Biotechnology Co., Ltd. (Nanjing, China), introducing restriction sites *Kpn*I and *Sac*I between *arsR* and *arsC*; P*ars* is As promoter and regulatory sequence, and *gfp*, *arsR* and *arsC* are green fluorescent protein (GFP) gene (BAG13002.1), As regulatory protein (ArsR) gene (CAQ33820.1) and cytoplasmic As(V) reductase (ArsC) gene (CAQ33821.1), respectively. *E. coli* BL21(DE3) and *E. coli* JM109 were obtained from Tiangen Biochemical Technology Co., Ltd. (Beijing, China). As(V) ($Na_3AsO_4 \cdot 12H_2O$) and As(III) ($NaAsO_2$) were obtained from Merck (Darmstadt, Germany). As standards including As(V), As(III), dimethylarsinic acid (DMAs(V)) and monomethylarsonic acid (MAs(V)) were provided by the Institute of Urban Environment, Chinese Academy of Science. Unless specified otherwise, all chemicals noted in this article were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and with analytical or better grade.

2.2. Generation of phylogenetic tree of As efflux transporters

The $ArsB_{RP}$ (*R. palustris* CGA009), $ACR3_{RP}$ (*R. palustris* CGA009) and $ArsB_{EC}$ (*E. coli* BL21 (DE3)) amino acid sequence were aligned with by DNAMAN7 software (Lynnon Biosoft, Quebec, Canada). Phylogenetic tree based on the results from alignment was constructed using NJ method in MEGA 7.0 software (Filipski et al., 2014).

2.3. Construction and verification of recombinant E. coli AW3110 strains

The $arsB_{RP}$ (WP_011157811.1), $acr3_{RP}$ (WP_042441188.1) and $arsB_{EC}$ (BX571963.1) were amplified by PCR. Three primer pairs were designed by Oligo 7.0 software (Molecular Biology Insights, Inc., Plymouth, MN, USA) (Table 1). The *arsB*/*acr3* fragment and pUC19-P*ars-gfp-arsRC* plasmid were double-digested by *Kpn*I and *Sac*I and were recovered by agarose gel electrophoresis. The gene fragments were ligated with pUC19-P*ars-gfp-arsRC* plasmid at 16 °C for 20 h and transformed into *E. coli* JM109 competent cells. The cells were plated on Luria-Bertani (LB) solid medium containing 100 μg/mL ampicillin and incubated overnight at 37 °C. Positive clones were individually picked to identify the recombinant plasmid (pUC19-P*ars-gfp-arsRB*$_{RP}$C, pUC19-P*ars-gfp-arsR-acr3*$_{RP}$-*arsC* and pUC19-P*ars-gfp-arsRB*$_{EC}$C, respectively) by double enzyme digestion with *Kpn*I and *Sac*I to obtain the cloned As efflux protein genes. The identified recombinant plasmids were then transformed into competent *E. coli* AW3110(DE3) cells. The engineered *E. coli* AW3110 harboring recombinant plasmids were screened for ampicillin resistance and verified by double enzyme digestion and sequencing by Suzhou Genewiz Biotechnology Co., Ltd. (Suzhou, China), named as *E. coli* AW3110($ArsB_{RP}$), *E. coli* AW3110($ACR3_{RP}$), *E. coli* AW3110($ArsB_{EC}$) and *E. coli* AW3110(pUC19-P*ars-gfp-arsRC*), respectively.

PCR-amplified *arsM* (BX571963.1) derived from *R. palustris* CGA009 was substituted for the *arsC* in the identified recombinant plasmid above (pUC19-P*ars-gfp-arsRB*$_{RP}$C, pUC19-P*ars-gfp-arsR-acr3*$_{RP}$-*arsC*, pUC19-P*ars-gfp-arsRB*$_{EC}$C and pUC19-P*ars-gfp-arsRC*) and transformed into competent *E. coli* JM109 cells. The positive clones were verified by PCR. The identified plasmids (pUC19-P*ars-gfp-arsRB*$_{RP}$*M*, pUC19-P*ars-gfp-arsR-acr3*$_{RP}$-*arsM*, pUC19-P*ars-gfp-arsRB*$_{EC}$*M* and pUC19-P*ars-gfp-arsRM*, respectively) were then transformed into competent *E. coli* AW3110(DE3) cells and verified by PCR and sequencing by Suzhou Genewiz Biotechnology Co., Ltd. (Suzhou, China), named as *E. coli* AW3110($ArsB_{RP}$M), *E. coli* AW3110($ACR3_{RP}$M), *E. coli* AW3110($ArsB_{EC}$M) and *E. coli* AW3110(ArsM), respectively.

Moreover, since the recombinant plasmid contained *gfp*, the recombinant *E. coli* AW3110 strains mentioned above were all induced by As(III) to generate fluorescence that could further verify the recombinant strains. The bacterial suspension (OD_{600} about 0.15) of recombinant *E. coli* AW3110 (containing 100 g/mL ampicillin) were induced by 2.0 μmol/L As(III) at 200 r/min for 12 h at 30 °C. Control experiments without As(III) were performed with addition of the same amount of sterilized deionized water under the same conditions. The appropriate amount of bacterial solution was diluted to OD_{600} of 0.1 with LB liquid medium. The fluorescence intensity of GFP was measured by fluorescence spectrophotometer (F-320, Tianjin Gangdong Sci & Tech. Development Co., Ltd., Tianjin, China). The relevant parameters were set as listed below: 479 nm excitation wavelength, 516 nm emission wavelength, 479–600 nm spectral scanning range, 10.0 nm excitation slit, 5.0 nm emission slit, 400 V PMT voltage, 1200 nm/min scanning speed, 2.0 s sensitivity.

Table 1
Primers used in this study.

Genes	Gene lengths (bp)	Primer sequence (5'→3')	Enzyme site	T_m (°C)
$arsB_{RP}$	1300	F: CGGGTACCATGCTGATCGCGCTCGCTATCTTT	*Kpn*I	69.2
		R: CGGAGCTCTTACAGACTGAGCCTTATGGCGAG	*Sac*I	69.2
$acr3_{RP}$	1069	F: CGGGTACCATGTCCACCTTCGAACGCTACCT	*Kpn*I	69.3
		R: CGGAGCTCTCAGCCCGGCGTCCGCGCCAC	*Sac*I	76.7
$arsB_{EC}$	1306	F:CAGGTACCATGTTACTGGCAGGCGCTATCTTTGT	*Kpn*I	67.7
		R:CGGAGCTCTTACAAAGTGAAAGAGAGACGTAGCG	*Sac*I	67.7
$arsM_{RP}$	847	F: ACGGAGCTCATGCCCACTGACATGCAAGACG	*Sac*I	69.6
		R: GCGGAATTCTCACCCGCAGCAGCGCGC	*Eco*RI	73.7

2.4. Determination of As resistance in the recombinant E. coli AW3110 strains

The recombinant *E. coli* AW3110 strains (OD_{600} about 0.15) were incubated in 10 mL LB medium at 200 r/min, 37 °C for 12 h. *E. coli* AW3110($ArsB_{RP}$), *E. coli* AW3110($ACR3_{RP}$), *E. coli* AW3110($ACR3_{RP}$) and the control (*E. coli* AW3110(pUC19-P*ars-gfp-arsRC*)) were cultured at final concentrations of 0.5, 1.0, 3.0, 5.0, 8.0 mmol/L As(III) or 0.05, 0.1, 0.5, 1, 2, 4, 8, 32 mmol/L As(V); while *E. coli* AW3110($ArsB_{RP}$M), *E. coli* AW3110($ACR3_{RP}$M), *E. coli* AW3110($ArsB_{EC}$M) and the control *(E. coli* AW3110(ArsM)) were cultured at 2, 10, 25, 50, 100, 200, 500, 1000, 2000, 3000 and 4000 μmol/L As(III). Control experiments without As(III) were carried out under the same conditions. Each experiment was performed in triplicate. Bacterial growth was monitored by measuring OD_{600} and the growth inhibition rate was calculated. The As concentration was logarithmically plotted against the growth inhibition rate. The data was analyzed by OriginPro 8.0 software (Origin Lab Corporation, MS, USA) to calculate the semi-inhibitory concentration of As on growth (IC_{50}). The bacterial resistance capacity was estimated as IC_{50}.

2.5. Determination of fluorescence intensity of the recombinant E. coli AW3110 strains

The recombinant *E. coli* AW3110 strains with OD_{600} of 0.15 were incubated with As(III) in 10 mL LB medium containing 0, 0.5, 1.0, 2.0, 3.0 and 5.0 μmol/L As(III) at 200 r/min for 12 h at 30 °C. Bacterial culture medium was diluted until reaching an OD_{600} of about 0.1 with LB liquid medium. The fluorescence density of samples was detected by fluorescence spectrophotometer (F-320, Tianjin Gangdong Sci & Tech. Development Co., Ltd., Tianjin, China) with parameters of 479 nm excitation wavelength and emission wavelengths of 516 nm.

2.6. As speciation analysis of the recombinant E. coli AW3110 strains

The recombinant *E. coli* AW3110 strains at an OD_{600} of 0.15 were separated into 5 mL LB liquid medium containing 25 μmol/L As(III) or As(V) and incubated at 200 r/min for 12 h at 30 °C. At the same time, the control experiments without As carried out. 1.00 mL of the sample was digested with 10 mL of 1% (V/V) HNO_3 with a microwave digestion apparatus (MASTER 40A, Sineo Microwave Chemistry Technology Co., Ltd, Shanghai, China) for microwave-assisted digestion. The digestion parameters were set as described previously (Zhu et al., 2008). The digested sample was centrifuged at 4 °C 8000×*g* for 5 min to collect the supernatant. The supernatant was filtered through a 0.22 μm membrane (Millipore, Bedford, MA, USA), and analyzed by high-performance liquid chromatography (HPLC) (Agilent 1200, Agilent, USA) coupled with inductively-coupled plasma mass spectrometry (ICP-MS) (Agilent 7700cx, Agilent, USA) using previously established instrument parameters (Yin et al., 2011). The mobile phases consisted of 10.0 mmol/L of $NH_4H_2PO_4$ and 10.0 mmol/L of NH_4NO_3 at pH 9.25. As speciation in the samples was identified by retention time to the mixed standards including As(III), As(V), MAs(V) and DMAs(V) whose concentration of As in each form is 10 μg/L. The As was quantified by external calibration curves with peak areas.

2.7. As volatilization by E. coli AW3110 strains co-expressing arsB/acr3 and arsM

The difference in total concentration of As before and after incubation with As(III) can be used to illustrate the level of volatilization. Digestion of 1.00 mL of bacterial solution after incubation for 12 h. The digestion method was performed according to GB/T 5009.17—2014 with modification (Tang et al., 2018), and the total As was determined by atomic fluorescence spectrometer (AFS-8220, Beijing Jitian Instrument Co., Ltd., Beijing, China). This digesting system was provided with a 60 mA As lamp, −270 V high pressures, 300 mL/min carrier gas flow, 200 mL/min auxiliary gas flow, 200 °C atomizer temperature, 10 s sampling time, and 5% HCl mobile phase.

3. Results

3.1. Phylogenetic analysis of As efflux transporters

The amino acid sequence of ArsB and ACR3 efflux transporters from different microbes were selected as reference for phylogenetic analysis with $ArsB_{RP}$ (*R. palustris* CGA009), $ACR3_{RP}$ (*R. palustris* CGA009) and $ArsB_{EC}$ (*E. coli* BL21(DE3)) (Fig. 1) that were used in this study, and the three As efflux transporters were aligned (Supplementary Fig. S2). The three As efflux transporter sequences showed significant differences as shown in the phylogenetic tree and in the sequence alignment. $ArsB_{RP}$ was closely related to $ArsB_{EC}$ (74%) and distantly related to $ACR3_{RP}$ (33%). Considering the differences among $ArsB_{RP}$, $ACR3_{RP}$ and $ArsB_{EC}$, we hypothesize that they may contribute differently to As resistance and As efflux activity.

3.2. Construction and identification of two types of the recombinant E. coli AW3110 strains

The recombinant *E. coli* AW3110 strains expressing genes encoding different As efflux transporters ($arsB_{RP}$, $acr3_{RP}$ and $arsB_{EC}$) were constructed using molecular cloning techniques. The recombinant plasmids (pUC19-P*ars-gfp-arsRB*$_{RP}$*C*, pUC19-P*ars-gfp-arsR-acr3*$_{RP}$*-arsC* and pUC19-P*ars-gfp-arsRB*$_{EC}$*C*) were identified by double digestion with *Kpn*I and *Sac*I, and the $arsB_{RP}$ (1300 bp), $acr3_{RP}$ (1069 bp) and $arsB_{EC}$ (1306 bp) fragments were identical in size to the target fragment (Supplementary Fig. S3A). Further sequencing results showed that $arsB_{RP}$, $acr3_{RP}$ and $arsB_{EC}$ were identical to the

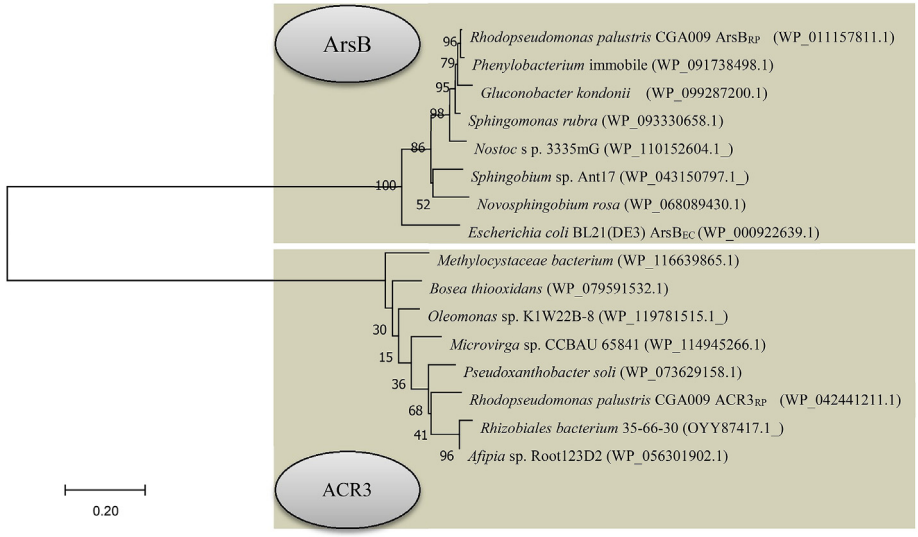

Fig. 1. Phylogenetic relationships of $ArsB_{RP}$, $ACR3_{RP}$ and $ArsB_{EC}$. A neighbor-joining phylogenetic tree of sequences was built using MEGA 7.0. Accession numbers of this sequence are provided in parentheses. The bold denoted sequence was investigated in this study.

sequence deposited in GenBank.*arsM* (BX571963.1) derived from *R. palustris* CGA009 was amplified by PCR and then was substituted for *arsC* in the identified recombinant plasmid described above, and transformed into *E. coli* AW3110(DE3) to obtain recombinant strains co-expressing *arsB*/*acr3* and *arsM*, and their recombinant plasmid was isolated and the cloning steps by PCR (Supplementary Fig. S3B). The size of the detected PCR product shown was the same as that of the target gene (847 bp). The sequencing results showed that the *arsM* sequence expressed in the recombinant strains was identical to the sequence deposited in GenBank.

Since *gfp* was inserted downstream of the *ars* operon genes, the fluorescence intensity produced by GFP in recombinants could further verify the construction of recombinants mentioned above and the expression of the *ars* operon. In the absence of As(III), the recombinant *E. coli* AW3110 strains produced negligible fluorescence at 516.6 nm; while the recombinant *E. coli* AW3110 strains produced strong fluorescence when induced with 2.0 μmol/L As(III) (Supplementary Fig. S4). The results indicated that these recombinant plasmids (pUC19-P*ars-gfp-arsRB*$_{RP}$*C*/*M*, pUC19-P*ars-gfp-arsR- acr3*$_{RP}$*-arsC*/*M* and pUC19-P*ars-gfp-arsRB*$_{EC}$*C*/*M*) were induced by As(III) in *E. coli* AW3110(DE3).

3.3. As resistance of recombinant E. coli AW3110 strains

The resistance to As(V) and As(III) of recombinant *E. coli* AW3110 strains expressing *arsB*/*acr3* and the control (*E. coli* AW3110(pUC19-P*ars-gfp-arsRC*)) without *arsB*/*acr3* were determined (Fig. 2). The inhibition of growth in the four recombinant *E. coli* AW3110 strains gradually increased with growing As(V) or As(III) concentration. However, the degree of growth inhibition of the different recombinant *E. coli* AW3110 strains displayed significant differences. In the presence of As(V), the IC_{50} of the four recombinant *E. coli* AW3110 strains were 274.82 ± 1.48 μmol/L (the control), 7201.12 ± 3.24 μmol/L (*E. coli* AW3110($ArsB_{RP}$)), 396.05 ± 1.25 μmol/L (*E. coli* AW3110($ACR3_{RP}$)) and 4355.12 ± 3.18 μmol/L (*E. coli* AW3110($ArsB_{EC}$)), respectively, indicating that the order of the recombinant strain's As(V) resistance was *E. coli* AW3110($ArsB_{RP}$) > *E. coli* AW3110($ArsB_{EC}$) > *E. coli* AW3110($ACR3_{RP}$) > the control; similarly, their As(III) resistance decreased significantly in the presence of As(III), and the order of As(III) resistance was consistent to the order displayed by As(V) (Supplementary Table S1). It could be observed that the As resistance of the recombinants expressing *arsB*/*acr3* significantly increased but varied greatly compared to the control, indicating that they showed different As efflux activities with the order in which they conferred resistance was $ArsB_{RP}$ > $ArsB_{EC}$ > $ACR3_{RP}$.

Compared to the recombinant *E. coli* AW3110 strains expressing *arsB*/*acr*3, the IC_{50} of the recombinant *E. coli* AW3110 strains co-expressing *arsB*/*acr3* and *arsM* further increased (Fig. 3), indicating that the introduction of *arsM* enhanced the As(III) resistance of the recombinant *E. coli* AW3110 strains co-expressing *arsB*/*acr3* and *arsM*, and their order of As(III) resistance was similar to the recombinant *E. coli* AW3110 strains expressing *arsB*/*acr3*. This could be attributed to ArsM converting As(III) into methylated As species with a lower toxicity and volatile TMAs(III). In addition, due to all As efflux transporters of each recombinant *E. coli* AW3110 strains co-expressing *arsB*/*acr3* and *arsM* being different, the rate of As(III) efflux was different, causing the varied content of As(III) methylated by ArsM, so their IC_{50} displayed great differences.

3.4. Comparison of intracellular As(III) concentration in different recombinant E. coli AW3110 cells

As(III) is a derepression of the ArsR regulated *ars* operon. Since *gfp* was inserted downstream of the *ars* operon and the fluorescence intensity produced by GFP can be regarded as measurement of the concentration of As(III) in cells qualitatively. As(III) in the cell initiates the expression of the *ars* promoter by binding to the ArsR repressor leading to derepression which makes the expression of its downstream genes possible. In the range of 0–2.0 μmol/L As(III), the fluorescence intensity of GFP encoded by *gfp* increased with increasing As(III) concentration, which showed a linear relationship (Fig. 4 and Supplementary Table S2). The higher the activity of As efflux transporters, the lower the intracellular As(III) concentration in the cell, which caused a better correlation between As(III) concentration and detected fluorescence intensity; therefore, the activity of the As efflux transporters could be determined by the size of the linear slope, and the obtained results were correlated to the results of As resistance assay. Compared to the recombinant *E. coli* AW3110 expressing only *arsB*/*acr3*, the recombinant *E. coli* AW3110 co-expressing *arsB*/*acr3* and *arsM* displayed a lower fluorescence intensity, indicating that a lower concentration of As(III) was

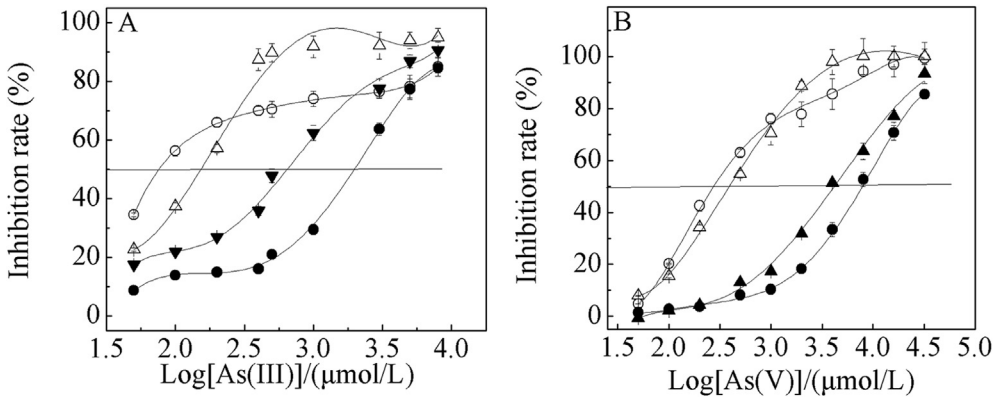

Fig. 2. The effect of As(III) (A) and As(V) (B) on the growth inhibition rate of recombinant *E. coli* AW3110 strains bearing vector plasmid pUC19-P*ars-gfp-arsRC* (○), pUC19-P*ars-gfp-arsRB*$_{RP}$*C* (●), pUC19-P*ars-gfp-arsR-acr3*$_{RP}$*-arsC* (□) and pUC19-P*ars-gfp-arsRB*$_{EC}$*C* (■). The recombinant *E. coli* AW3110 strains expressing *arsB*/*acr*3 (OD_{600} about 0.15) and the control (*E. coli* AW3110 (pUC19-P*ars-gfp-arsRC*)) were incubated in 10 mL LB medium at 200 r/min, 37 °C for 12 h. The bacterial resistance capacity was estimated as IC_{50}. Error bars indicate the standard deviation from three independent experiments

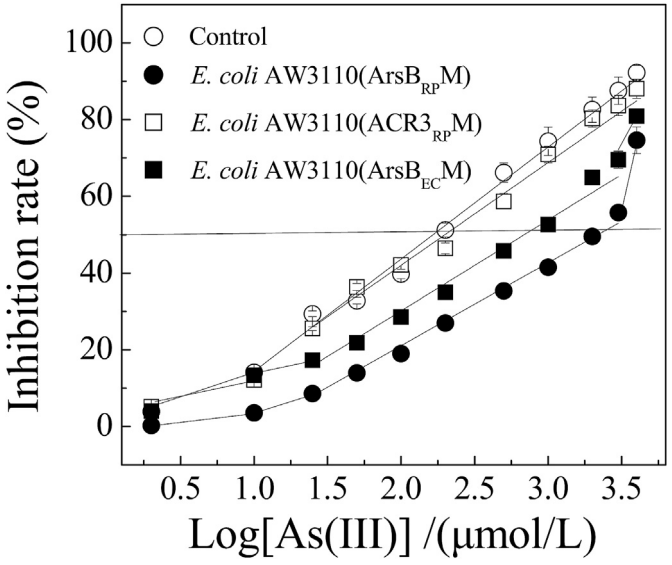

Fig. 3. The effect of As(III) on the growth inhibition rate of recombinant *E. coli* AW3110 co-expressing *arsB*/*acr*3 and *arsM*. The recombinant strains (OD_{600} about 0.15) were incubated in 10 mL LB medium at 200 r/min, 37 °C for 12 h. The control is recombinant *E. coli* AW3110 strain bearing vector plasmid pUC19-P*ars-gfp-arsRM* (no *arsB* or *acr*3). The bacterial resistance capacity was estimated as IC_{50}. Error bars indicate the standard deviation from three independent experiments.

present in the cells, which was caused by As methylation and volatilization.

3.5. As volatilization rate by recombinant E. coli AW3110 strains co-expressing arsB/acr3 and arsM

To examine the effect of different As efflux transporters on the As volatilization rate of recombinant *E. coli* AW3110 strains co-expressing *arsB*/*acr3* and *arsM*, the difference in total As concentration before and after bacterial growth was used to assess the As volatilization rate (Fig. 5). After incubation for 12 h at 25.0 μmol/L As(III) with *E. coli* AW3110(ArsM) containing no *arsB*/*acr3*, *E. coli* AW3110(ArsB$_{RP}$M), *E. coli* AW3110(ACR3$_{RP}$M) and *E. coli* AW3110(ArsB$_{EC}$M), the total As content in the medium decreased by 9.42%, 4.93%, 8.17% and 6.82%, respectively; while incubation at 100.0 μmol/L As(III) under the same conditions, the total As content decreased by 10.01%, 3.99%, 8.20% and 5.65%, respectively. These results showed that the As volatilization rate of recombinant *E. coli* AW3110 strains co-expressing *arsB*/*acr3* and *arsM* was decreased compared to that of the control (*E. coli* AW3110(ArsM)), suggesting that the presence of As efflux transporters limited the As volatilization rate of the different recombinant strains. The expression of different As efflux transporters and co-expression with *arsM* showed significant differences in As volatilization rate, indicating that the As efflux rates of individual As efflux transporters were different, with ArsB$_{RP}$ exhibiting the highest efflux rate, followed by ArsB$_{EC}$, and ACR3$_{RP}$ having the lowest rate.

3.6. As speciation in recombinant E. coli AW3110 cells

Since recombinant plasmid (pUC19-P*ars-gfp-arsRB*$_{RP}$*C*, pUC19-P*ars-gfp-arsR-acr3*$_{RP}$*-arsC*, pUC19-P*ars-gfp-arsRB*$_{EC}$*C* and pUC19-P*ars-gfp-arsRC*) contains a gene encoding an ArsC. ArsC can reduce As(V) to As(III) which can then be pumped out of the cell by an As efflux transporter. Therefore, adding As(V) into the medium could further compare the activity of the individual As efflux transporters by determining the extracellular concentration of As(III). After incubation for 12 h at 25.0 μmol/L As(V), the As(III) in vitro was determined by HPLC-ICP-MS (Fig. 6A). By comparing the peak area of As(III), the As efflux capacity of As efflux transporters displayed the order: ArsB$_{RP}$ > ArsB$_{EC}$ > ACR3$_{RP}$.

When the recombinant *E. coli* AW3110 co-expressing *arsB*/*acr3* and *arsM* were exposed to 25.0 μmol/L As(III) for 12 h, As speciation in the medium was determined (Fig. 6B). MAs(V) and DMAs(V) were detected by bacteria co-expressing *arsB*/*acr3* and *arsM*, but their total accumulated concentration did not change significantly, while the As(III) concentration showed a major decrease. The residual As(III) in the medium was *E. coli* AW3110(ArsB$_{RP}$M) > *E. coli* AW3110(ArsB$_{EC}$M) > *E. coli* AW3110(ACR3$_{RP}$M) > *E. coli* AW3110(ArsM), further indicating that As efflux proteins could significantly limit the As volatilization rate.

4. Discussion

Arsenic (As) methylation is regarded as an efficient way to remediate As contamination due concomitant volatilization of As (Wang et al., 2014b). However, most microorganisms with the ability to methylate As did not show a high rate of As volatilization (Wang et al., 2014a). Currently, the As geochemical cycle driven by microbes and the bioremediation of As contaminated environments by employing microbe-mediated As transformations,

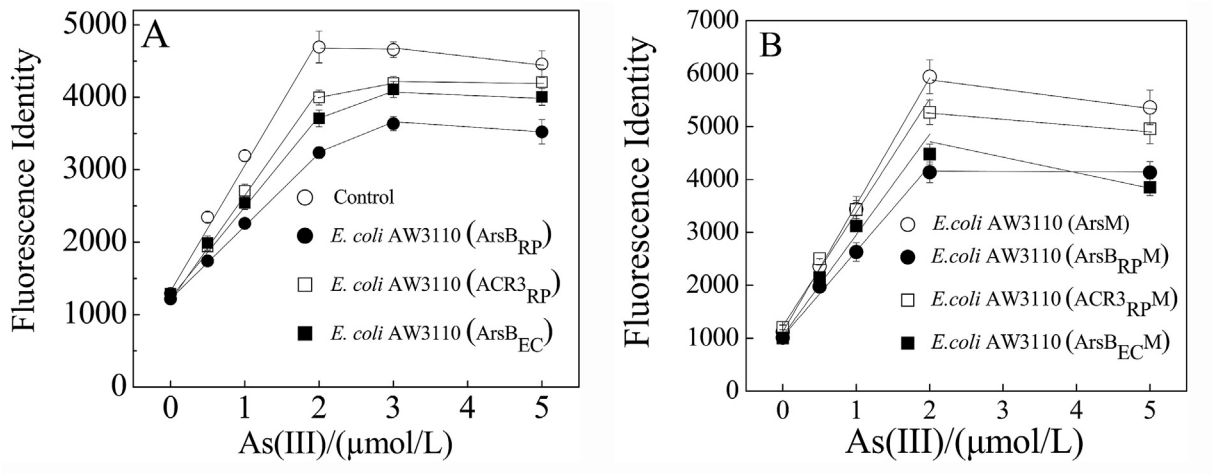

Fig. 4. Fluorescence detection of culture medium (50-fold dilution) of the recombinant *E. coli* AW3110 strains expressing *arsB*/*acr*3 (A) and co-expressing *arsB*/*acr*3 and *arsM* (B). The control is *E. coli* AW3110(pUC19-P*ars-gfp-arsRC*) containing no *arsB* or *acr*3. Error bars indicate the standard deviation from three independent experiments

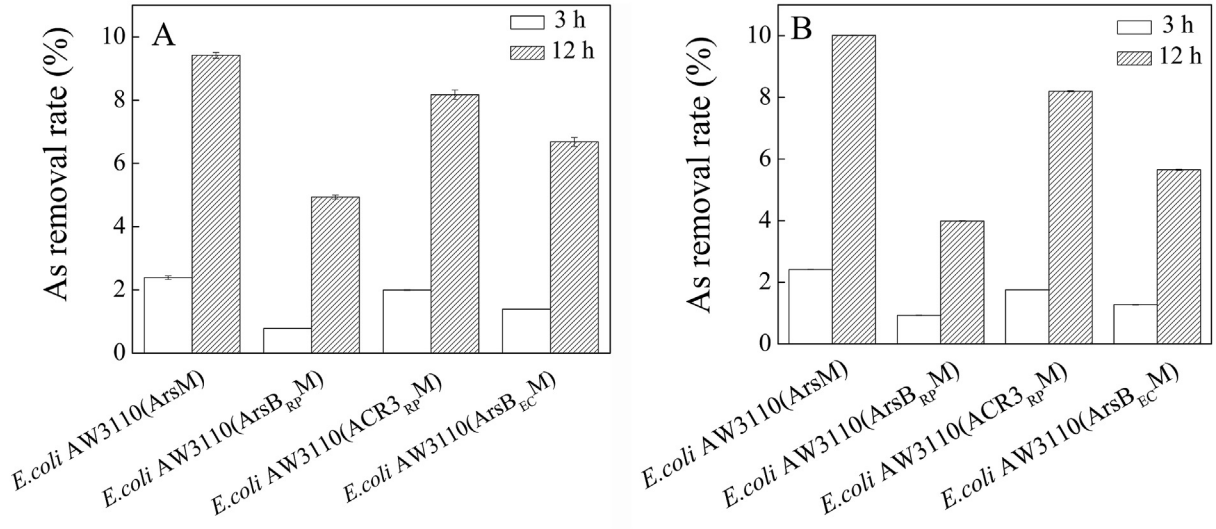

Fig. 5. Determination of As volatilization rate of the recombinant *E. coli* AW3110 strains co-expressing *arsB*/*acr*3 and *arsM* at 25.0 μmol/L (A) and 100.0 μmol/L (B) As(III).

including As methylation, have received increased attention. Therefore, it is important to explore ways to accelerate the As volatilization rate of microorganisms for remediation of As-contaminated soil or water.

Arsenic efflux systems are found in nearly every organism and evolved to rid cells of the toxic metalloid (Garbinski et al., 2019). Inorganic As detoxification in prokaryotes has been well described (Ben Fekih et al., 2018). *arsB*/*acr3* are ubiquitous in microorganisms and the encoded gene products play an important and even decisive role in conferring As resistance in microorganisms (Ben Fekih et al., 2018). The *arsB*/*acr3* encoded transporters pump As(III) out of the cell in a strain encoding functions for both As efflux and methylation, which reduces the substrate concentration available for ArsM. Therefore, the presence of *arsB*/*acr3* is bound to limit microbial As methylation efficiency. In this study, we revealed that $ArsB_{RP}$ and $ACR3_{RP}$ from *R. palustris* CGA009 not only displayed different As efflux rate, but also limited the As volatilization rate. Thus, As efflux was shown to be the preferred pathway of As resistance in microorganisms where As efflux and As methylation pathway coexist, and As efflux is a significant internal factor limiting As volatilization. However, recent studies have showed that the methylation pathway is nearly ubiquitous in global *Prochlorococcus* populations; while the complete efflux pathway is only maintained in populations which experience extremely low PO_4:AsO_4 (Saunders and Rocap, 2016), meanwhile, the methyl group of DMAs(V) produced by *Prochlorococcus* released into the environment could become substrates that could be exploited by *Pelagibacter*, producing a flow of energy to P-limited ocean regions (Giovannoni et al., 2019). Therefore, microorganisms may be affected by environmental factors so that they choose As efflux pathway thereby reducing the substrate concentration of As methylation, thereby lowering As volatilization efficiency.

Previous studies have looked at As methylation at the genetic (Qin et al., 2006) and transcriptional (Zhao et al., 2015) level when examining *arsM* in *R. palustris* CGA009, a good model organism for studying As detoxification. This has inspired us to examine whether the co-expression of *arsM* and *arsB*/*acr3* may show a negative effect on As methylation. To test this hypothesis, *arsM* and *arsB*/*acr3* derived from *R. palustris* CGA009 were co-expressed in *E. coli* AW3110(DE3). The results showed that either ArsB or ACR3

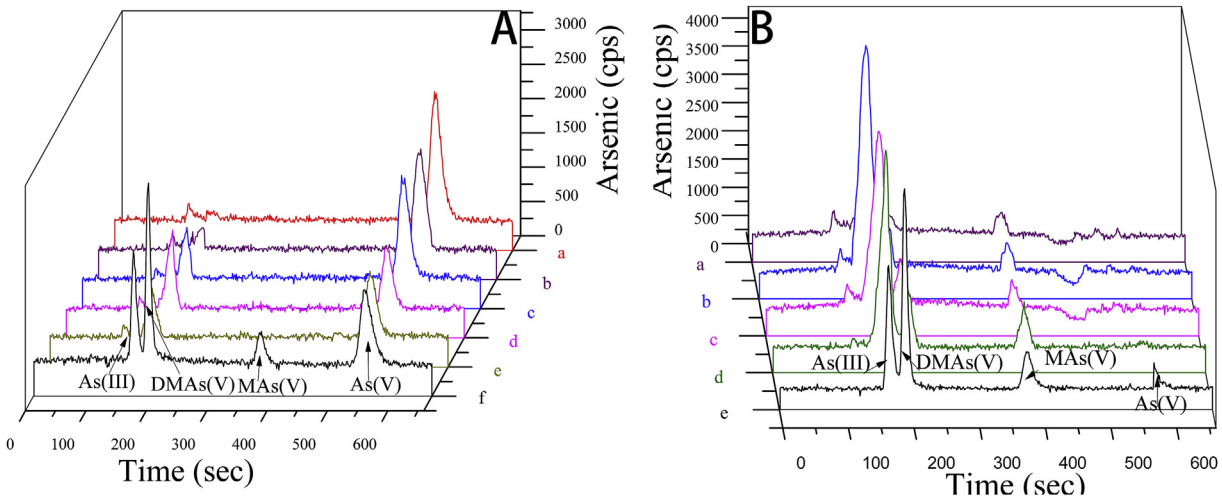

Fig. 6. Determination of As speciation in recombinant *E. coli* AW3110 cells by HPLC-ICP-MS. (A) Determination of extracellular As(III) of recombinant *E. coli* AW3110 strains expressing *arsB*/*acr*3. a~f indicate *E. coli* AW3110 (the blank), *E. coli* AW3110(pUC19-P*ars-gfp-arsRC*), *E. coli* AW3110($ArsB_{RP}$), *E. coli* AW3110($ACR3_{RP}$), *E. coli* AW3110($ArsB_{EC}$) and As standards, respectively. Speciation of As of the recombinants grown on 25.0 μmol/L As(V) for 12 h were detected using supernatant of bacterial suspension. (B) As speciation in recombinant *E. coli* AW3110 strains co-expressing *arsB*/*acr*3 and *arsM*. ãe indicate *E. coli* AW3110(ArsM), *E. coli* AW3110($ArsB_{RP}$M), *E. coli* AW3110($ACR3_{RP}$M), *E. coli* AW3110($ArsB_{EC}$M) and As standards, respectively. Chromatogram of the standards including As(III), DMAs(V), MAs(V) and As(V) whose concentration of As in each form is 10 μg/L. Speciation of As of the recombinants grown on 25.0 μmol/L As(III) for 12 h were detected using bacterial suspension.

transported As(III) across the cytoplasmic membrane into the periplasmic space, and both transporters competed with ArsM over As(III), thereby limiting the As methylation efficiency of ArsM. Different As efflux transporters had varied effects on As methylation efficiency. $ArsB_{RP}$ had the greatest effect on As volatilization with a rate reaching only 3.99% compared to the control (*E. coli* AW3110(ArsM)) (10.01%) when incubated for 12 h at a concentration of 100.0 μmol/L As(III), and followed by $ArsB_{EC}$ (5.65%) and $ACR3_{RP}$ (8.20%). Therefore, in a follow-up study, the *arsB*/*acr3* of *R. palustris* CGA009 will be deleted or inactivated to improve the As methylation efficiency, and to provide an efficient bioremediation tool able to remove As(III) from the environment.

This study established a microbial As biosensor for monitoring intracellular As concentration to compare the As efflux activities of three As efflux transporters and investigate the interaction between *arsB*/*acr3* and *arsM* for the first time. Compared to the reported As biosensors for detecting As in vitro (Bereza-Malcolm et al., 2018; Kaur et al., 2015; Kumar et al., 2016), the As biosensor in this study harbored the *arsR* whose product could selectively bind with As(III) as shown in previous studies (Ke et al., 2018; Li et al., 2015; Yang et al., 2013) could specifically detect As(III) using the fluorescence intensity produced by GFP in vivo. This is a novel method that could be applied to determinate intracellular As concentrations in microorganisms. However, in this study, we only employed this method to detect intracellular As content qualitatively. Therefore, in later studies, we intend to improve our method to quantitatively detect the intracellular As content, thereby improving the application value of this method.

Arsenic methylation in bacteria has been studied in increasing detail, but factors that limit the rate of As methylation in vivo such as As efflux have not been studied in great detail. *E. coli* AW3110(ArsM) without gene encoding an As efflux transporter displayed the highest As volatilization efficiency, which was consistent to results of previous studies on *Arsenicibacter rosenii* SM-1 with a high As volatilization efficiency lacking an As efflux protein (Huang et al., 2016) and an engineered strain expressing *arsM* in *E. coli* AW3110 (Ke et al., 2019). Compared to *E. coli* AW3110 containing *arsM* derived from *R. palustris* CGA009 (Ke et al., 2019; Yuan et al., 2008), the recombinant *E. coli* AW3110 strains co-expressing *arsB*/*acr3* and *arsM* had a lower As volatilization efficiency. This difference appeared to be due to the existence of As efflux transporters (ArsB/ACR3), which reduced the As volatilization rate. The As(III) resistance of recombinant *E. coli* AW3110 strains co-expressing *arsB*/*acr3* and *arsM* to As (III) was higher than the resistance of strains containing only *arsB*/*acr3* or *arsM*, but lowered activity of As methylation than strains expressing only *arsM*, indicating that As efflux may be the primary pathway in *R. palustris* CGA009 rather than As methylation. The low As volatilization was here shown to be due to the presence of genes encoding both As efflux transporters and ArsM, when these genes were co-expressed, the As efflux transporters competitively pump the As(III) out of the cell, resulting in a decreased concentration of the substrate for As methylation and thereby decreasing microbial As methylation and subsequent As volatilization. In addition to genes encoding As efflux transporters, other genes associated with reducing intracellular As concentration (genes encoding aquaglyceroporins, As(III) oxidase), genes encoding transporters responsible for efflux of methylated As metabolites (e.g. MMAs(V) or DMAs(V)) or reducing the product concentration (e.g. demethylase gene) may also reduce the rate of As volatilization.

Collectively, this study provided a new method to detect intracellular As using an As biosensor to compare the As efflux activities of As efflux transporters and investigate the interaction between *arsB*/*acr3* and *arsM* for the first time. The study proposed As efflux transporters might be a significant factor limiting As methylation efficiency for those microorganisms encoding both As efflux and methylation pathway, which provided an explanation for the low methylation efficiency of microorganisms and an effective strategy for screening microorganisms with a higher As volatilization rate to be an ideal toolbox for bioremediation of As-contaminated environments.

Competing financial interests

The authors declare they have no actual or potential competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.124822.

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