Appl Microbiol Biotechnol (2006) 73:332–338
DOI 10.1007/s00253-006-0483-6

BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Whole-cell arsenite biosensor using photosynthetic bacterium *Rhodovulum sulfidophilum*

Rhodovulum sulfidophilum as an arsenite biosensor

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Received: 2 March 2006 / Revised: 20 April 2006 / Accepted: 21 April 2006 / Published online: 30 May 2006

Abstract An arsenite biosensor plasmid was constructed in *Escherichia coli* by inserting the operator/promoter region of the ars operon and the *arsR* gene from *E. coli* and the *crtA* gene, which is responsible for carotenoid synthesis in the photosynthetic bacterium, *Rhodovulum sulfidophilum*, into the broad-host-range plasmid vector, pRK415. The biosensor plasmid, pSENSE-As, was introduced into a *crtA*-deleted mutant strain of *R. sulfidophilum* (CDM2), which is yellow in culture due to its content of spheroiden (SE) and demethylspheroidene (DMSE). CDM2 containing pSENSE-As changed from yellow to red by the addition of arsenite, which caused enzymatic transformation of SE and DMSE to spheroidenone (SO) and demethylspheroidenone (DMSO). Reverse transcriptase PCR analysis showed that the color change depended on transcription of the *crtA* gene in pSENSE-As. The color change could be clearly recognized with the naked eye at 5 μg/l arsenite. The biosensor strain did not respond to other metals except for bismuth and antimony, which caused significant accumulation of SO and DMSO in the cells at 60 and 600 μg/l, respectively. This biosensor indicates the presence of arsenite with a bacterial color change without the need to add a special reagent or substrate for color development, enabling this pollutant to be monitored in samples by the naked eye in sunlight, even where electricity is not available.

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Introduction

As an application of photosynthetic bacteria, we sought to construct a whole-cell biosensor using the bacteria as the host and a pigment gene as a novel reporter to detect environmental pollutants with high sensitivity. This biosensor would indicate the presence of a pollutant by a bacterial color change, without the need to add a special reagent or substrate for color development. Moreover, no equipment for detecting fluorescence or chemiluminescence under light-shielded conditions would be necessary to detect the pollutant, which could be monitored in samples by the naked eye under sunlight, even in areas where electricity is not available. Various biological systems have been used to construct whole-cell bacterial sensors (Belkin 2003; D'Souza 2001). The sensor bacteria are usually genetically engineered to contain a reporter plasmid that carries a gene(s) and/or elements that respond to target chemicals. Several reporter genes, such as firefly or bacterial luciferase (Wilson and Hastings 1998), green fluorescent protein (GFP) from *Aequorea victoria* (Tsien 1998), and *lacZ* (Stocker et al. 2003), have been used for monitoring environmental pollutants. Most studies have been conducted using *Eschericha coli*, for which host–vector systems are well developed, as the host. Recently, cyanobacteria have been used as reporter strains for assessing nitrogen bioavailability in freshwater lakes (Gillor et al. 2003). Hence, a variety of sensor strains are needed to develop versatile whole-cell sensor systems.

In this study, we used a photosynthetic bacterium, *Rhodovulum sulfidophilum*, for the sensor strain, and the

Table 1 Primers used in this study

Primer	Sequence	Restriction enzyme
term 1	5′-GGA*AAGCTT*GCGCCGCGCGGGCTCCTCCC-	*Hin*dIII
	CGCGCGGCGCTTTTTTTCT*CTGCAG*AA-3′	*Pst*I
term 2	5′-TT*CTGCAG*AAAAAAAGCGCC-3′	*Pst*I
crtArv1	5′-AACGTCGAGCTTGTCGATGA-3′	
crtArv2	5′-TTCC*TCTAGA*CGGTTTAGGAGAGTCGCAAA-3′	*Xba*I
crtArv3	5′-AA*GGTACC*AGAAGGGAAAGGGTTGAGTC-3′	*Kpn*I
Pars-S1	5′-GC*TCTAGA*TTACCTTCCTCTGCACTTAC-3′	*Xba*I
Pars-A1	5′-GC*TCTAGA*TAGTGTCTCTCTTCGAAGCG-3′	*Xba*I
arsR-A2	5′-GC*TCTAGA*TTAACTGCAAATGTTCTTACTGTCCCC-3′	*Xba*I
crtA-RT-S1	5′-TATGACAACTGGGCTTCGCG-3′	
crtA-RT-A1	5′-GGTCTGGAGTCCGATTTCAGC-3′	
arsR-RT-S1	5′-CTGGGCATCGTTTTACTGCTCAGC-3′	
arsR-RT-A1	5′-CGGAACAGTTTTGTCGAGCC-3′	
rRNA-RT-S1	5′-AGCCTACGATCTATAGCTGG-3′	
RRNA-RT-A1	5′-GGAATTCCTCTCGGAAAACC-3′	

Recognition sites for restriction enzymes are italicized

crtA gene, which is responsible for carotenoid synthesis, as a novel reporter gene, to construct a highly sensitive whole-cell biosensor for detecting arsenite. *Rhodovulum sulfidophilum* belongs to a group of purple photosynthetic bacteria that synthesize carotenoids through the spheroiden (SE) pathway (Takaichi 2001; Takaichi et al. 2001). In this pathway, SE is formed by O-methylation of the C-1 hydroxy group of demethylspheroidene (DMSE) by O-methyltransferase (CrtF), and spheroidenone (SO) is formed by ketolation at the C-2 of SE monooxygenase (CrtA). SO is the predominant carotenoid under semi-aerobic conditions. CrtA in these bacteria is responsible for changing the color of the cultures from yellow, due to SE, to red, due to SO (Yeliseev et al. 1996). In our previous study we isolated a CrtA-deleted mutant of *R. sulfidophilum* (CDM2) by inserting a kanamycin cassette; the mutant accumulates SE, and therefore displays a yellow color in culture (Maeda et al. 2005).

Arsenic contamination in ground water is a serious problem in countries, such as India and Bangladesh, that rely on millions of tubewells for their drinking water supply (Nordstrom 2002). A simple and sensitive arsenite sensor system is needed to prevent arsenite-derived diseases in these communities. We sought to construct a whole-cell biosensor using CDM2 that could detect the presence of arsenite by bacterial color change. The arsenite-resistance mechanism has been extensively analyzed in *E. coli*. The ars operon, consisting of two regulatory genes (*arsR* and *arsD*) and three structural genes (*arsA*, *arsB*, and *arsC*), is responsible for arsenite resistance (Wu and Rosen 1991; Kaur and Rosen 1992). ArsR is a repressor that binds the operator/promoter region of the ars operon (*OPars*) and prevents transcription of the downstream genes for arsenite resistance. In the presence of arsenite, ArsR binds with arsenite, which causes a conformational change and its dissociation from the operator (Shi et al. 1996). We constructed a biosensor plasmid containing the promoter-less *crtA* gene derived from *R. sulfidophilum* and the arsenite-responding DNA fragment containing *OPars* and *arsR* derived from *E. coli*. The color change from yellow to red in CDM2 cultures containing this recombinant plasmid could be clearly recognized by the naked eye at the final concentration of more than 5 μg/l arsenite.

Materials and methods

Reagents

Standard solutions (1,000 mg/l) of As_2O_3, $Bi(NO_3)_3$, Cd, Co, Ni, $Pb(NO_3)_2$, $SbCl_3$, and $Zn(NO_3)_2$ were purchased from Nacalai Tesque (Kyoto, Japan). All chemicals were reagent grade and all solutions were prepared using deionized (Ultrapure water system, Barnstead International, Dubuque, IA, USA) distilled water. Luria–Bertani medium (LB) (Sambrook et al. 1982) was purchased from Nacalai Tesque (Kyoto, Japan).

Bacteria and growth conditions

CDM2, which is a *crtA*-deleted mutant strain of *R. sulfidophilum*, *E. coli* JM109 (Yanisch Perron et al. 1985), *E. coli* S17-1 (λpir) (Simon et al. 1983), and *E. coli* K12 MG1655 (ATCC47076) were used. CDM2 was cultivated in modified Okamoto medium (MOM) (Maeda et al. 2003) in a cultivation vessel with a screw-on cap under an incandescent lamp at a photon flux intensity of 40 to 45 μmol sec^{-1} m^{-2} at 25 °C. *Escherichia coli* strains were

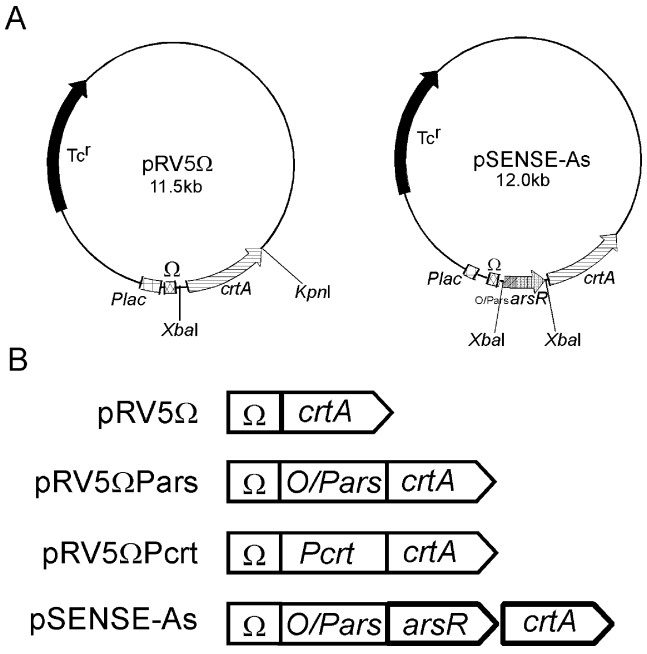

Fig. 1 Schematic representation of plasmids and DNA regions inserted into the multicloning site of pRK415. Promoter-less and arsenate sensor plasmids are designated as pRV5Ω and pSENSE-As, respectively (**a**). Four DNA regions (**b**) were inserted into pRK415 to evaluate the terminator sequence, *O/Pars*, and *arsR*. *Tc^r^* tetracycline resistance, *Amp^r^* ampicillin resistance, *Ω* terminator sequence

cultivated in LB. Antibiotics, when necessary, were added to *E. coli* culture at final concentrations of 50 μg/ml ampicillin, 30 μg/ml kanamycin, and 25 μg/ml tetracycline, and to *R. sulfidophilum* CDM2 culture at final concentrations of 30 μg/ml kanamycin and 5 μg/ml tetracycline.

Construction of a sensor plasmid in *E. coli*

A broad-host-range plasmid vector pRK415 (Keen et al. 1988) was used for the construction of the sensor plasmid. Because the *lac* promoter is located upstream of the multicloning site in pRK415, we inserted a terminator sequence between the *lac* promoter and the multicloning site to prevent the transcription of inserted genes from the *lac* promoter. The terminator region was amplified by PCR using *R. sulfidophilum* genome DNA as the template and primers term 1 and 2 (Table 1), designed using the terminator sequence of *pufC* gene from the same bacterium (Masuda et al. 1999). The amplified DNA was inserted into the *Hin*dIII/*Pst*I site of pRK415 to form pRKΩ. The *crtA* gene was amplified by PCR from *R. sulfidophilum* genome DNA using primers crtArv1 and 3, and cloned into pGEM-T Easy to form pGV2. The cloned DNA encompassed the region from about 300 bp upstream of the initiation codon to 30 bp downstream of the stop codon of *crtA*. This sequence contained the promoter region of *crtA* (*Pcrt*). A promoter-less *crtA* fragment was then amplified by PCR using pGV2 as the template and primers crtArv2 and 3, digested with *Xba*I/*Kpn*I, and cloned into pRKΩ to form pRV5Ω (Fig. 1a).

To examine whether the *E. coli O/Pars* could be recognized by RNA polymerase in CDM2, we inserted *E. coli O/Pars* between the Ω and *crtA* open reading frame (ORF) in pRV5Ω. *O/Pars* was amplified by PCR using *E. coli* K12 genome DNA as the template and primers ParsS1 and ParsA1 (Table 1) and cloned into pGEM-T Easy to form pGPars. The inserted DNA was cut with *Xba*I and inserted into an *Xba*I site between the Ω and *crtA* ORF sequence in pRV5Ω to form pRVΩPars (Fig. 1b). The arsenite-responding DNA fragment containing *O/Pars* and *arsR* was amplified by PCR using *E. coli* K12 genome DNA as the template and primers Pars-S1 and arsR-A2 (Table 1) and cloned into pGEM-T Easy to form pGASK. The inserted DNA was cut with *Xba*I and inserted into the *Xba*I site between the Ω and *crtA* ORF sequence in pRV5Ω to form pSENSE-As. All the cloning procedures described above were performed using *E. coli* JM109. The DNA manipulations and PCR were performed according to standard protocols (Sambrook et al. 1982) or the manufacturers' instructions.

Conjugal transfer of plasmids

The recombinant plasmids derived from pRK415 were introduced into *E. coli* S17-1 and then transferred into CDM2 by conjugation. *Escherichia coli* containing recombinant plasmid was cultured in LB containing 25 μg/ml tetracycline in a test tube, and CDM2 was cultured in MOM containing 30 μg/ml kanamycin in a Roux bottle under light. Conjugal transfer was performed by the method described by Yun et al. (1990). The conjugants were spread onto MOM-agar plates containing 30 μg/ml kanamycin and 5 μg/ml tetracycline, and incubated for a few days under light conditions.

Quantitative analysis of carotenoids

CDM2 containing recombinant plasmid was lyophilized overnight, and the pigments were extracted with a mixture of acetone and methanol (7:2, v/v) by vortexing for 2 min. After the cell debris was removed by filtration, the extract was analyzed using a high-performance liquid chromatography (HPLC) system equipped with a μBondapak C_{18} column (3.9×300 mm, 125-Å pore size, Nihon Waters, Tokyo, Japan), as described previously (Takaichi et al. 2001).

Statistics

The data were analyzed for statistical significance using Student's *t*-test.

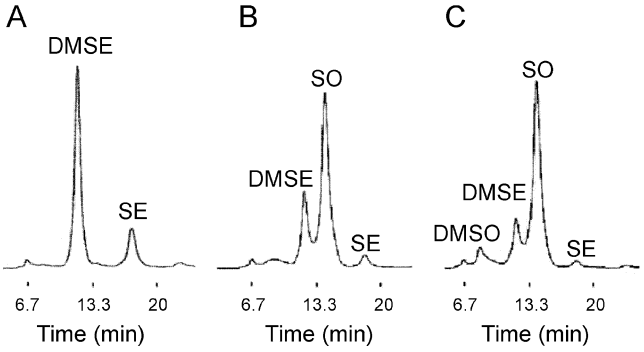

Fig. 2 HPLC profiles of the carotenoids synthesized by recombinant CDM2 strains. CDM2 was transformed with pRV5Ω (**a**), pRV5ΩPars (**b**), and pRV5ΩPcrt (**c**). *SE* spheroidene, *DMSE* demethylspheroidene, *SO* spheroidenone, *DMSO* demethylspheroidenone

Results

Construction of arsenite sensor plasmid

We first searched for a DNA region similar to *arsR* in the *R. sulfidophilum* genome by southern hybridization and PCR amplification, using the sequences derived from *E. coli arsR*. Because we could not find such a region, we used the *O*/*Pars* and *arsR* region derived from *E. coli*. A recombinant plasmid, pRV5ΩPars, which contained the *E. coli O*/*Pars* sequence upstream of *crtA*, was constructed and introduced into CDM2, as described in "Materials and methods." When cultured in MOM under light, CDM2 containing the promoter-less plasmid pRV5Ω retained its yellow color. There was no SO accumulation in these cells, as shown in Fig. 2a, indicating that the terminator (Ω) sequence successfully prevented the transcription of *crtA* from the *lac* promoter located upstream of the pRK415 multicloning site. The promoter of *crtA* from *R. sulfidophilum* was then inserted into pRV5Ω to form pRV5ΩPcrt. CDM2

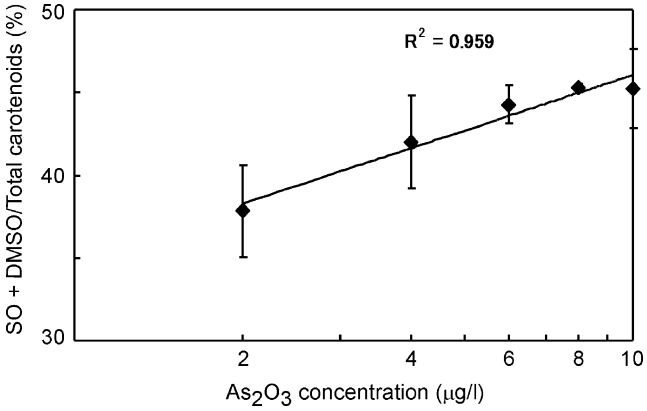

Fig. 3 Dose-dependent accumulation of SO and DMSO by arsenite. CDM2 containing pSENSE-As was cultured in the presence of arsenite, and the percentage of SO and DMSO in the total carotenoids was calculated from the pigment analysis by HPLC. The numbers at abscissa axis were the final concentrations of arsenite in the culture medium. Values are the means±SD of three independent experiments

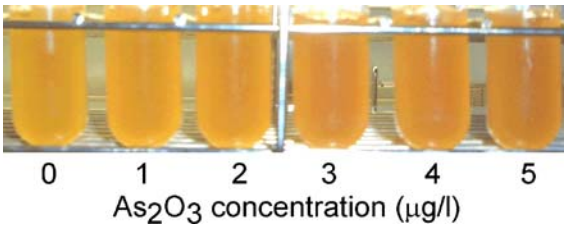

Fig. 4 Change of color in the arsenite sensor strain in response to the addition of low concentrations of arsenite. The initial cell concentration (OD660) was adjusted to 0.5, and cells were cultivated under light in the presence of various concentrations of arsenite for 24 h. The photograph was taken 24 h after the addition of arsenite. The indicated numbers were the final concentrations of arsenite in the culture medium

containing pRV5ΩPcrt accumulated SO and demethylspheroidenone (DMSO) and showed a decrease in SE, the substrate of CrtA, and DMSE, the precursor of SE in the SE pathway (Fig. 2c). These cells appeared red in culture. Similarly, CDM2 containing pRV5ΩPars accumulated SO and showed decreases in SE and DMSE (Fig. 2b). These results indicate that the *E. coli O*/*Pars* could be recognized by *R. sulfidophilum* RNA polymerase, and that the transcription was not suppressed by an endogenous protein in CDM2. The strain appeared to have no arsR-like protein. All the cultures in Fig. 2 were incubated without arsenite. We then constructed the sensor plasmid, pSENSE-As, which contained the *O*/*Pars* and *arsR* region derived from *E. coli* between the Ω and *crtA* ORF.

Evaluation of the sensor strain

The sensor plasmid, pSENSE-As, in *E. coli* S17-1 was transferred into CDM2 by conjugation. The sensor strain, CDM2(pSENSE-As), was then cultured in the presence of various concentrations of arsenite under light conditions for 24 h. Figure 3 shows the ratio of SO and DMSO, which are responsible for the red color, to the total amount of carotenoids. Because the ratio was dose-dependently increased by the addition of arsenate, *crtA* in pSENSE-As appeared to be transcribed and translated responding to arsenite at the final concentration of less than 10 μg/l. As shown in Fig. 4, the bacterial color change could not be recognized by the naked eye at concentrations of less than 2 μg/l arsenite. The slight and clear color changes were recognized at the concentrations of 3 and 5 μg/l arsenite, respectively. Therefore, the lower limit of the arsenite detection by the naked eye appeared to be 3 μg/l in this biosensor. Next, we examined the effect of high concentrations of arsenite on the bacterial color (Fig. 5). The addition of 6,000 μg/l arsenite showed the toxic effect on the sensor strain, and even the yellow color could not be seen. There was little difference between the color in the presence of 60 and 600 μg/l arsenite. The SO and DMSO

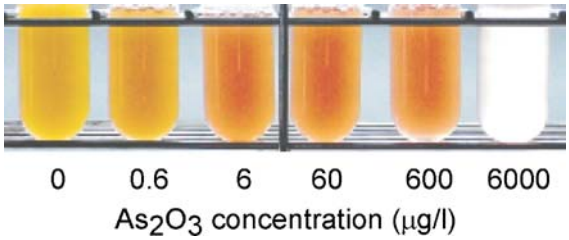

Fig. 5 Change of color in the arsenite sensor strain in response to the addition of high concentrations of arsenite. The initial cell concentration (OD660) was adjusted to 0.5, and cells were cultivated under light in the presence of various concentration of arsenite for 24 h. The photograph was taken 24 h after the addition of arsenite. The indicated numbers were the final concentrations of arsenite in the culture medium

increased to about 60% of the total carotenoids at 60 μg/l arsenite and remained at 60% as the arsenite was increased to 600 μg/l (data not shown).

The total RNAs were extracted from CDM2(pSENSE-As) cultured in the absence or presence of 60 μg/l arsenite, and reverse transcriptase (RT)-PCR analysis was carried out using primers designed for *crtA*, *arsR*, and rRNA (Fig. 6). In the absence of arsenite, only a faint band of *crtA* transcript was detected. In contrast, the transcription of *crtA* was markedly induced by the addition of arsenite. These results indicate that the expression of red color was dependent on the transcription of the *crtA* gene on pSENSE-As. Although the RT-PCR product of the *arsR* gene was also significantly increased by the addition of arsenite, a certain level of *arsR* transcript was also observed in the absence of arsenite. This result suggests that the suppression at the *O*/*Pars* region was not so strict that it prevented the synthesis of an appropriate amount of ArsR to inhibit transcription in the absence of arsenite.

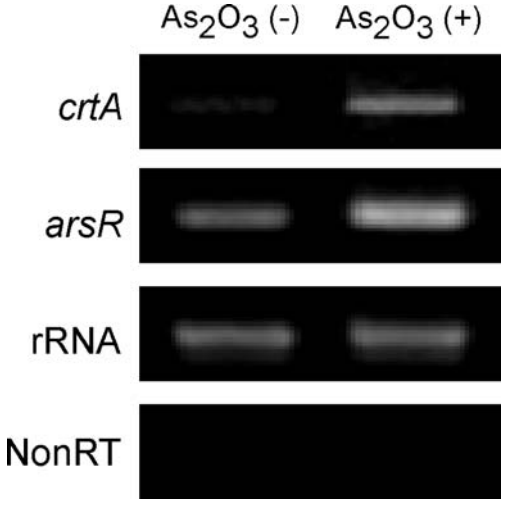

Fig. 6 RT-PCR analysis of the transcription of the *crtA* and *arsR* genes. CDM2 containing pSENSE-As was cultured for 24 h in the absence and presence of 60 μg/l arsenite, then the total RNA was extracted and used for RT-PCR analysis

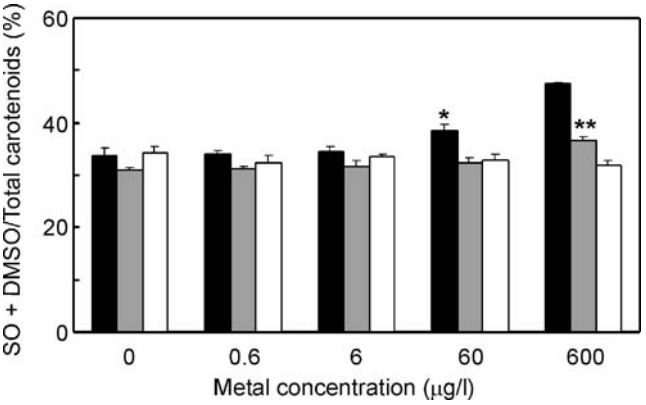

Fig. 7 Response of the biosensor strain to various metals. CDM2 containing pSENSE-As was cultured for 24 h in the presence of Bi $(NO_3)_3$ (*closed bars*), $SbCl_3$ (*gray bars*), or a mixture of $FeCl_2$, $CuSO_4$, $AlK(SO_4)_2$, and $MnCl_2$ (*open bars*) at the final concentrations indicated. In the metal mixture, each metal was added at the final concentrations indicated. *N.T.* not tested. Values are the means±SD of three experiments. *Asterisks* indicate a significant difference between the values from cells cultured in the absence and presence of arsenite or bismuth (**P<0.01, *P<0.05)

Sensitivity to bismuth and antimony

We next examined the sensitivity of CDM2(pSENSE-As) to other metals. Various metals were tested at concentrations of 0.6–6,000 μg/l. Among the metals tested, CDM2 (pSENSE-As) only responded to bismuth and antimony (Fig. 7). Significant accumulation of SO and DMSO was observed at 60 μg/l bismuth and 600 μg/l antimonite. Because 0.6 μg/l arsenite yielded a significant increase in SO and DMSO contents (data not shown), the concentration eliciting the reaction was 100- to 1,000-fold higher than that of arsenite. The CDM2(pSENSE-As) did not respond at all to a mixture of $FeCl_2$, $CuSO_4$, $AlK(SO_4)_2$, and $MnCl_2$. A cytotoxic effect on the sensor strain was observed at 6,000 μg/l metal, at which concentration the determination of pigments could not be carried out. The addition of cadmium, cobalt, lead, nickel, and zinc separately at 0.6–6,000 μg/l did not affect the percentage of SO and DMSO at all (data not shown).

Discussion

Very few attempts have been made to construct a whole-cell biosensor using a host other than *E. coli*. In this study, an arsenite sensor plasmid was constructed for the photosynthetic bacterium *R. sulfidophilum* using the broad-host-range vector pRK415, containing (1) a novel reporter, which was the *R. sulfidophilum* gene responsible for carotenoid synthesis, and (2) the DNA region responsible for arsenite resistance in *E. coli*. The sensor strain CDM2(pSENSE-As) clearly indicated the presence of 5 μg/l arsenite by a color

change from yellow to red in a test tube of the culture. The permissive concentration of arsenite in drinking water ranges from 10 to 50 μg/l, depending on the country. Hence, this sensor system appears to be sensitive enough for practical use. For example, when we want to detect arsenite at a concentration of higher than 50 μg/l by using this biosensor, 1 ml of test sample is added to 9 ml of the medium containing the sensor strain. A color change from yellow to red indicates the presence of hazardous amounts of arsenite in the test sample. Although a remarkable change of the color could not be recognized in the range of 60 to 600 μg/l arsenite, this biosensor can be used to examine whether the test sample contains hazardous concentrations of arsenite or not. Moreover, in our preliminary experiments, a small sample of the strain spotted on filter paper turned red in response to the addition of arsenite solution, indicating that this sensor system could probably be applied to a field test kit for arsenite detection. When the pigment composition in the sensor strain was analyzed by HPLC, the presence of arsenite was detected after 3 h of incubation (data not shown). However, it took 12–24 h to detect arsenite by the naked eye, without using a sensing device. Existing *E. coli*-based biosensors using the lux, GFP, or *lacZ* genes allow detection in less than a few hours. The time required for the naked-eye detection using CDM2(pSENSE-As) might be shortened by using a high-copy-number plasmid in place of pRK415, which has a copy number of two to four (Keen et al. 1988). However, a high-copy-number plasmid that can be replicated in photosynthetic bacteria is not available at present. Further research is necessary to develop an appropriate host–vector system for photosynthetic microorganisms.

The biosensor cross-reacted with bismuth at 60 μg/l. Bismuth is in the same element family as arsenic, and is known to induce the ars operon in *E. coli* and cyanobacterium *Synechocystis* sp., although to a lesser extent than arsenite (Wu and Rosen 1993; Lopez Maury et al. 2003). However, the concentration eliciting a visible reaction from yellow to red was more than 600 μg/l (data not shown). The biosensor was less sensitive to antimony than to bismuth. Therefore, the biosensor constructed in this study could be used for arsenite-specific purposes.

The regulatory region of the *ars* operon, *O*/*Pars*, is not strictly regulated by ArsR, because a certain amount of *arsR* gene transcription is necessary to make the protein that represses the operon in the absence of arsenite. There is a stem-loop structure in the mRNA 10-bp downstream of the *arsR* stop codon and upstream of the *crtA* gene. This structure might have a mild transcription terminator function to stop the transcription of genes downstream of the *arsR* gene in the absence of arsenite. Because our sensor plasmid contained this stem-loop structure, it might explain why only a small amount of *crtA* transcript was observed in the absence of arsenite. It is possible that ArsR detached from *O*/*Pars* in the presence of arsenite to activate the transcription of *arsR* and *crtA*, at a level that overcame the effect of the terminator-like sequence. There might be another possibility that the stem-loop structure protected *arsR* mRNA against the degradation by exoribonucleases from 3′ end of *crtA* mRNA.

Various biosensors using CDM2 could be constructed by inserting DNA that responds to a specific substance between the Ω and *crtA* ORF in pRV5Ω. The strain CDM2 was isolated from marine photosynthetic bacterium, *R. sulfidophilum*, with the aim of developing a biosensor detecting the pollution of seawater, as well as freshwater. Moreover, at the time of commercialization of the biosensor, the marine strain could expect to possess the resistance to various stresses including osmotic change caused by freeze-drying. We have prepared biosensor strains responding to dimethyl sulfide (DMS) using the promoter region of the DMS dehydrogenase gene (Maeda et al. 2006). DMS is known as the source of the smell of the sea, which determines the quality of lavers used in Asian cooking. Thus, CDM2-based biosensors could be applied in a variety of fields, including aquaculture and environmental biotechnology.

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