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Title

A dual system using compartmentalized partnered replication for selection of arsenic-responsive transcriptional regulator

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Running Title

A dual system for selection of ArsR

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Abstract:

Engineering and design of genetic circuit in living cell is critical in accessing the beneficial application of synthetic biology. Directed evolution can avoid the complicated rational design of such circuit by screening or selecting functional circuit from non-functional one. Here we proposed a positive-negative selection system for selecting a transcription factor that activates gene expression in response to arsenic in solution. First, we developed a whole cell biosensor for sensing arsenite in liquid using a regulator (ArsR) and a reporter (GFP), and evaluated its performance. Second, we developed a positive selection system for active ArsR using compartmentalized partnered replication that uses thermostable DNA polymerase as the reporter of activity. Third, we developed a negative selection system using sucrose-induced suicide gene SacB as the reporter for exclusion of inactive ArsR variants.

Keywords: arsenic contamination; biosensor; gene circuit; water-in-oil emulsion.

Abbreviations: As(III), arsenite; As(V), arsenate; CPR, compartmentalized partnered replication; CSR, compartmentalized self-replication; GFP, green fluorescence protein.

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Introduction

Arsenic contamination in groundwater is a harmful threat to million people worldwide (1-6). Arsenic exists under the form of various chemical species in different physicochemical behavior, toxicity, bioavailability and biotransformation. Among various arsenic species, arsenite [As(III)] and arsenate [As(V)] are the most toxic forms and regarded as human carcinogenic substances (4). The tolerable weekly intake for inorganic arsenic is set at 15 μg/kg of body weight, and the highest acceptable level of arsenic in drinking water is 10 μg/L (7). Unfortunately, populations in the most contaminated area have least access to the centralized drinking water system. Moreover, arsenic analysis of environmental samples requires sophisticated chemical equipment and technical expertise that leads to become detecting the level of arsenic in potable water a troublesome in these regions. To address this problem, scientists have been keen on developing biosensors for arsenic as an alternative solution (8-13).

Due to more simplicity and lower cost in relative to the analytical instruments, biosensors have been paid attention in fieldworks requiring *in situ* detection. However, the deployment of biosensors is limited in term of operational lifetimes and their sensitivity to other environmental factors such as temperature and pH. These problems are the obstacle to use biosensors in place of conventional analytical instruments. Since biosensors are beneficial in measuring the contaminant concentration in places where the access to the instruments and technical experts are out of reach, solution of these problems should be contributed to environmental assessments such as surveillance of arsenic contamination in groundwater.

Biosensors using whole microbial cells to determine a variety of heavy metals have been reported (10,14-16). During the course of evolution, bacteria have acquired mechanisms to resist many toxic metals. Many of these heavy metal resistance systems have been studied to their genetic determinants such as mercury (17), zinc (18), cobalt (19), cadmium (20), lead (20) and arsenic (14-16). Arsenic resistance gene in bacteria, *ars* operon, has been studied widely (21-23). The main genes of arsenic resistant system include the transcriptional repressor ArsR, the arsenic efflux pump ArsB, and the arsenate reductase ArsC (24). The *ars* genes have been found in chromosomal locations

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or plasmid-encoded in a number of Gram-negative bacteria as well as in Gram-positive. The minimal number of gene in one operon is *arsRBC* in the chromosome of *E. coli* (25).

In this study, we developed a whole cell biosensor for arsenite by coupling the *arsR* gene with a reporter gene under arsenite-dependent promoter, and designed a two-step selection system using compartmentalized partnered replication (CPR) (26,27) and a *sacB*-dependent sucrose sensitivity (28) toward its directed evolution. We evaluated the performance of the arsenite biosensor and confirmed feasibility of the two-step selection by proof-of-principle experiments. Our results provide a novel scheme for directed evolution of whole cell biosensors using pairs of transcriptional regulator and its responsive promoter.

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Materials and Methods

Bacterial strains and culture condition

Escherichia coli strain XL10-Gold and HST08 (Agilent Technologies) were used for all experiments of constructing plasmids and BL21(DE3) codon-plus RIPL (Stratagene) for protein expression. Bacterial cultures were grown at 37°C in 2×YT or LB media containing kanamycin (25 μg/ml) (Wako), ampicillin (100 μg/ml), or both according to the required condition. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma Aldrich) and sodium arsenate (Nacalai Tesque) for required concentration were added to the culture for induction purposes.

Construction of plasmids

All primer sequences used in this study are listed in Table I. All cloning was performed by isothermal assembly (29) after PCR amplification with primers that contain 15-20 bp overlapped regions. For each assembled reaction, equal molar volume of insert and vector PCR products were mixed with 2× Gibson assembly master mix (New England Biolabs) and incubated at 50°C for 15 min. All transformation was performed by heat shock method using 42°C for 45 sec. For dual plasmids transformation to obtain whole-cell biosensor, *E. coli* BL21(DE3) codon-plus RIPL cell carrying the first plasmid was beforehand prepared to be chemically competent followed by transformation with the second plasmid. Positive colonies were checked by colony-direct PCR with primers ACYC-Up1 and T7R targeting both inserts in each plasmid.

For construction of pET-*arsR*, pET29 vector (Novagen) linearized by pET29-Up and -Down was ligated with an *arsR* gene amplified from *E. coli* K12 strain using the primers arsR-F and -R. pET-*Tth* was recently constructed (30).

pACYC-*gfp* was constructed as follows. Ars operator/promoter sequence, a *gfp* gene, and pACYCDuet1 vector (Novagen) were first amplified by arsO/P-F and -R, sfGFP-F and -R, and pACYC-Up1 and -Down1, respectively, and three sequences were ligated by the Gibson assembly. After ligation, the chloramphenicol resistance gene in pACYC was replaced with the ampicillin resistance gene from pUC19 using primers

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pUC19-F and -R, and pACYC-Up2 and -Down2. Then, T7 promoter from resulted plasmid was removed by using primers pACYC-Up3 and -Down3.

pACYC-*Tth* and pACYC-*sacB* were constructed using primers pACYC-Up4 and -Down5 for the vector, and Tth-F and -R for a modified *Tth* DNA pol I gene with a C-terminal HA-tag (YPYDVPDYA) coding sequence as recently constructed (30) or sacB-F and -R for *sacB* gene from pKM154 (Addgene), respectively. The null plasmid pACYC was constructed by deleting the *gfp* gene from pACYC-*gfp* by primers pACYC-Up3 and -Down4.

Induction of biosensors and fluorescence measurements

Induction condition for both large culture volume (3-5 mL) and small culture volume (200 μL) was as follows. Single colony was picked and incubated overnight at 37°C. Overnight cultures were diluted to OD_{600} (~0.1) and incubated until OD becomes 0.4 and induced with required concentrations of IPTG and/or As(III) ($AsNaO_2$). GFP expression was measured on 10% SDS-PAGE gels with Molecular Imager FX (Bio-Rad) or on 96-well microtiter plates (Thermo Fisher Scientific) with TECAN Safire Fluorescence microplate reader (MTX Lab Systems) at excitation/emission wavelengths of 470 nm/520 nm.

Compartmentalized partnered replication (CPR selection)

CPR was performed essentially as described by Ellefson *et al*. (27). The pre-culture of the library was prepared in LB broth and incubated at 37°C overnight. Then, 100 μl of overnight culture was diluted in 2 mL of 2× YT and shaken 37°C for 1 h. When the culture entered the log-phase, the culture was induced with required concentration of arsenic and kept shaking for 4 h. Approximately 10^9 cells were mixed with 200 μl PCR reaction mixture that contains PCR buffer, dNTPs and primers targeting the *arsR* gene. The resulted aqueous phase was added into 600 μL of oil mixture at 20 sec interval while rotating at maximum speed in 4°C with additional spinning for 5 min. The resulted water in oil reaction mixture was transferred to PCR tubes and performed the PCR of 20 cycles (95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min/kb). After PCR, collection was done by centrifugation. In detail, emulsion-PCR products were

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transferred to 1.7 mL micro centrifuge tube and spun at 15000 rpm for 10 min. Upper layer above white film (oil phase) was discarded and washed with 1 mL mineral oil by repeating the centrifugation process. The remaining aqueous phase was spun for 20 min. Then, the aqueous phase was transferred to a new tube using gel loading pipette tip by penetrating the white film layer.

Removal of inactive arsR using Levansucrase activity (OFF selection)

The pET29-derived plasmid library was transformed into the strain having pACYC-*sacB*. The pre-culture of the collected transformants was prepared by incubating at 37°C overnight. This full-grown culture was mixed in 1/100 volume with fresh medium and shaken at 37°C for 6 h until the OD becomes 1. This culture was plated on the LB agar plates with or without 5% sucrose. The colonies grown on sucrose-containing medium were checked by colony-direct PCR with primers T7-promoter and T7-terminator.

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Results

Design of biosensor strain

First we constructed a whole-cell biosensor with GFP as a reporter to measure arsenic in a broth sample. This arsenite [As(III)] biosensor strain contains a double-plasmid system where the regulator *arsR* gene was placed under the control of T7 promoter on pET29 (pBR322 ori; kanamycin resistance) and the reporter *gfp* gene was placed under the control of As(III)-dependent *ars* operator/promoter (O/P) on a pACYCduet1 derivative (p15A ori; ampicillin resistance). We utilized BL21(DE3) as host organism with the double-plasmid system, pET-*arsR*/pACYC-*gfp* (Fig. 1). In theory, when there is no As(III), the ArsR repressor inhibits the reporter expression. Upon the arsenic arrival into the cell *via* phosphate channel (31-33), ArsR binds to As(III) releasing the promoter and leading downstream reporter expression (Fig. 1).

Characterization of biosensor strain

The response of the biosensor strain was confirmed in four separate induction conditions with As(III) and IPTG. In term of the above arsenic-responsive strain (pET-*arsR*/pACYC-*gfp*), only the As(III) induction gave the increase in GFP expression (Fig. 2A) and fluorescence (Fig. 2B) as expected. On the other hand, the IPTG induction showed no effect, suggesting leaky expression of ArsR on the pET vector. Thus, further experiments were performed without IPTG. As negative-control strains, ALWAYS-OFF strain (pET-*arsR*/pACYC; *gfp* was deleted from pACYC) and ALWAYS-ON strain (pET-*Tth*/pACYC-*gfp*; pET29 carry *Tth* instead of *arsR*) were also tested.

We also measured the fluorescence of three strains after induction with different amount of As(III). Upon induction with 10 μg/L arsenite, the arsenic responsive strain reached a little over 2 fold of background expression level (Fig. 2C). The fold of GFP intensity reached approximately 5.5 when the arsenic level increased to 100 μg/L. There was no effect of arsenic concentration upon the GFP level with the ALWAYS-ON and ALWAYS-OFF strains (Fig. 2C). It is somewhat strange that the response of ALWAYS-ON strain is lower than that of the sensor strain at concentrations of As(III) >20 ug/L: the modified *Tth* DNA polymerase expression may compete with or repress

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the GFP expression by unknown mechanisms. The GFP expression of the arsenic-responsive strain was also confirmed by visualizing the protein bands with fluorescence imager (Fig. 2D).

To elucidate the detection limit and time dependent response, fluorescence levels of the biosensor strain upon exposure to different concentrations of arsenic level ranging from 0, 5, 10, 25, 50, 75 and 100 μg/L were determined after 1, 2 and 4 h, respectively (Fig. 3A). Background fluorescence was still observable from the bacterial cell itself without arsenic presence. With the 1-h induction time, the fluorescence increased almost 2 fold while the 2-h induction reached to over 3.5 fold relative to uninduced cell at 10 μg/L. The fluorescence regression was not found to be linear with increased arsenic concentration with the 2-h induction measurements at over 10 μg/L of As(III). The fluorescence was found to decrease after 4 h, indicating that long-term exposure to arsenic is toxic to cells.

One of the concerns in applying whole cell biosensor in the practical field is the lifetime of the bacterial cell. Therefore, to monitor the lifetime of arsenic detecting strain in term of viability, log phase cultures without arsenite exposure were kept as broth culture and pellet at room temperature or 4°C. The biosensor activity of each culture during six consecutive days was checked by fluorescent levels induced with 10 μg/L As(III) for 3 h (Fig. 3B). Roughly, there was not much difference in the viability between different temperatures or between broth culture and pellet. The cell between first and second day showed similar response. The response significantly dropped on day 3 and cell became non-responsive from day 4 and onwards.

Next, we examined the response of the cell to some of the most common metals along with arsenic. As shown in Fig. 3C, the biosensor showed no response to other metals while showing high affinity to arsenite. This result indicates the specificity of the biosensor.

Strategy for 2-step selection of arsenic biosensor

Since the genetic circuit of arsenic regulatory system described above worked well with the *gfp* reporter (pET-*arsR*/pACYC-*gfp*), we moved on to set-up directed evolution system for *arsR* by compartmentalized partnered replication (CPR) (Fig. 4A). We

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adopted CPR as a positive selection system because it is beneficial for selection under toxic conditions such as arsenite and it does not require expensive apparatus such as flow cytometer for selection (27).

In CPR, separation of single cells by compartmentalization ensures the amplification of a partnered gene that can drive the expression of a thermostable DNA polymerase gene. We constructed a reporter plasmid pACYC-*Tth* using the modified *Thermus thermophilus* DNA polymerase quite recently developed in our laboratory (30) which show higher fidelity and extension efficiency than wild-type *Taq* DNA polymerase used in the original CPR (27). In this ON selection system, however, not only a mutated *arsR* gene with higher sensitivity against As(III) but also inactive genes that behaves as ALWAYS-ON strain can be also selected as false positive (Fig. 4A).

In order to exclude false positive clones from CPR, we applied levansucrase *sacB* gene from *Bacillus subtilis* that confers sucrose sensitivity in *E. coli* (34,35). *B. subtilis sacB* gene encodes the secreted enzyme levansucrase (EC 2.4.1.10) that catalyzes hydrolysis of sucrose and synthesis of levans, which are high-molecular weight fructose polymers (36). SacB expression in the presence of sucrose is lethal in gram-negative bacteria such as *E. coli* (37), although the mechanism behind this lethality is not fully understood. We constructed the pET-*arsR*/pACYC-*sacB* and pET-*gfp*/pACYC-*sacB* strains similar to the other two reporter systems. In theory, *sacB* will not be expressed in *E. coli* containing active *arsR* gene in the absence of As(III). In contrast, *sacB* will not be expressed in cells with inactive *arsR*. Consequently, in the presence of sucrose, false positive clones with inactive gene will be removed and active *arsR* variants can be enriched (Fig. 4B).

Proof-of-principle experiments for CPR selection

In order to confirm the performance of the strain with dual plasmids pET-*arsR*/pACYC-*Tth* according to arsenic induction (Fig. 4A), we performed PCR with arsenite-induced cells. Consequently, amplified product of target gene at 500 bp was observed as expected (Fig. 5A, bottom). This result was also confirmed by Western blotting of the HA-tagged DNA polymerase using anti-HA-tag antibody (Fig. 5A, top).

Next, as a proof-of-principle experiment of CPR selection, we created a model library by mixing the pET-*arsR*/pACYC-*Tth* strain and a strain harboring pET-*gfp* in

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which *arsR* was replaced with *gfp* at ratio of 1:10. The cell mixture was compartmentalized in water-in-oil emulsion. In theory (Fig. 4A), expression of *Tth* pol in the pET-*arsR*/pACYC-*Tth* strain depends on the relation between ArsR binding activity and arsenite concentration. In contrast, the pET-*gfp*/pACYC-*Tth* strain expresses *Tth* pol under any circumstances. Thus, without arsenite in the system, only the pET-*gfp*/pACYC-*Tth* strain produces DNA polymerase leading amplification of the target gene, *i.e*., *gfp*. As shown in Fig. 5B, the stronger amplification of *gfp* gene was observed under the emulsified and no arsenite condition as expected. The band intensity profiles suggested 15-fold enrichment by a single round of CPR selection. These proof-of-principle experiments indicated that *arsR* variant with higher sensitivity on arsenite or inactive *arsR* will be enriched by the positive selection system.

Proof-of-principle experiments for OFF selection

Since the positive selection system developed above also enriches inactive *arsR* variants, we next checked OFF selection system designed above. First, we confirmed the viability of pET-*arsR*/pACYC-*sacB* strain (Fig. 4B). When sucrose was not added in medium, arsenite addition did not affect the growth of cells. In contrast, under sucrose medium condition, arsenite addition that activates *sacB* expression induced cell lethality as expected (Fig. 6A). These results indicated that *arsR* activity is required for the viability of cells harboring pACYC-*sacB* in the sucrose medium.

With this result, we proceeded to the enrichment experiment for the counter selection. Similarly to the positive selection, a model library was prepared by mixing the pET-*arsR*/pACYC-*sacB* and pET-*gfp*/pACYC-*sacB* strains at ratio of 1:10. Colony-direct PCR with common primers for *gfp* and *arsR* showed the model library actually prepared (Fig. 6B). Next, the model library cells were plated on sucrose medium, and resultant colonies were randomly picked up to colony-direct PCR. In this case, it was expected that only the strain that repress *sacB* expression under no arsenite conditions formed single colonies. Thus, colonies derived from the pET-*arsR*/pACYC-*sacB* strain were expected to be enriched by the selection. As expected, majorities of colonies after the OFF selection were the pET-*arsR*/pACYC-*sacB* strain (Fig. 6C). As summarized in Fig. 6D, 37-fold enrichment of active *arsR* gene was observed over a single round of the

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selection. This result strongly supported that inactive *arsR* as the false positives of the CPR selection were efficiently eliminated through the OFF selection developed here.

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Discussion

Here we developed an arsenite biosensor strain and established a dual-selection system toward its directed evolution. The biosensor can be stored without any temperature control during at least 2 days, which may have sufficient tolerance for daily surveillance of environmental arsenite near human habitations. Selection for cells in the ON state was achieved by coupling the expression of a gene required for active gene amplification and that in the OFF state was coupled to cell viability. Although we only showed that separation of ON/OFF state by the selection system, we believe that *arsR* variants with higher activity can be selected because of the potential of CPR system (27). Thus, the dual system will be utilized to select *arsR* variants with increased ability that contributes to improvement of the arsenite biosensor in near future.

Although the dual selection system developed here focused on the ArsR system in *E. coli*, the system can be applied to any genetic circuit using pairs of transcriptional regulator and its elements to regulate gene expression levels. The dual selection system will be most useful in the initial stages of constructing complex circuits, especially when there is only a small functional fraction of a large pool of candidates. It will be also possible to select for specific circuit performance by adjusting the selection pressure. The simplicity of the strategy and strength of this selection method should facilitate optimization of genetic switches and circuits in the future.

Recently Li *et al*. reported an evolved arsenic biosensor with improved sensitivity to the arsenic *via* directed evolution using fluorescence activated cell sorting (FACS) (38). Although FACS is a powerful tool to screen a biosensor cells with GFP as a reporter, the CPR selection system based on DNA polymerase as a reporter have following advantages: (i) it is not need to determine an appropriate threshold to select mutants with a higher sensitivity from a DNA library for each evolutionary cycle and (ii) the highly expensive FACS instrument is not required. These features are very important for applying the dual-selection system to versatile usage as described above.

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Table I. The sequences of primers used in this study

Primer	Sequence (5' to 3')
ACYC-Up1	GGATCTCGACGCTCTCCCT
arsO/P-F	CATCACCACAGCCAGGATCCCATTCGTTAAGTCATATATGT
	TTTTGACTTATCCGCTTCGAAGAGAGA
arsO/P-R	AGTTGTTCTCCTTTACTCATATTGCGCTCCTGATTGTTGCAG
	GTAGTGTCTCTCTTCGAAGCG
arsR-F	TTTAAGAAGGAGATATACATATGTCATTTCTGTACCCATCC
arsR-R	GTGCTCGAGGGATCCACTGCAAATGTTCTTAC
pACYC-Down1	TCGAGTCTGGTAAAGAAACC
pACYC-Down2	TTTAGCTTCCTTAGCTCCTG
pACYC-Down3	TAGCGACTCCTGCATTAGGAAGGAGATATACCATGGGCA
pACYC-Down4	TTGCGACTCCTGCATTAGGTCGAGTCYGGTAAAGAAACC
pACYC-Down5	TCGAGTCTGGTAAAGAAACC
pACYC-Up1	GGATCCTGGCTGTGGTGATG
pACYC-Up2	TAATTTTTTTAAGGCAGTTA
pACYC-Up3	CCTAATGCAGGAGTCGCATA
pACYC-Up4	ATTGCGTCCTGATTGTTGC
pET29-Up	CATATGTATATCTCCTTCTTAAAGTTAAAC
pET29-Down	GGATCCCTCGAGCACCACC
pUC19-F	TAACTGCCTTAAAAAAATTATTACCAATGCTTAATCAGTG
pUC19-R	CAGGAGCTAAGGAAGCTAAAATGAGTATTCAACATTTCCG
sacB-F	GCAACAATCAGGAGCGCAATATGAACATCAAAAAGTTTGC
sacB-R	GGTTTCTTTACCAGACTCGATTATTTGTTAACTGTTAA
sfGFP-F	ATGAGTAAAGGAGAAGAACT
sfGFP-R	GGTTTCTTTACCAGACTCGATCATTTGTAGAGCTCATCCA
T7-promoter	TAATACGACTCACACTATAGGG
T7-terminator	GCTAGTTATTGCTCAGCGG
Tth-F	GCAACAATCAGGAGCGCAATTTAATACGACTCA
Tth-R	CAAAAAACCCCTCAAGACCC

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Figure legends

Fig. 1 Illustration of arsenic dependent repression and activation of gene expression by ArsR. ArsR represses transcription at P_{ars} in the absence of arsenite [As(III)] (left) while activates in the presence of As(III) (right).

Fig. 2 Performance analysis of biosensor strain. The synthetic circuit was confirmed by producing three different strains. The first strain is arsenic dependent and contains pET29-*arsR* and pACYC-*gfp*. ALWAYS-ON strain carries pET29-*Tth* and pACYC-*gfp*. ALWAYS-OFF strain consists of pET29-*arsR* and pACYC. (A,B) Fluorescence of each strain was monitored after inducing with sodium arsenate (50 μg/L) and IPTG (0.1 mM) at log phase. Uninduced culture was used to normalize data. The results were confirmed by (A) fluorescence imager to visualize the GFP bands after SDS-PAGE analysis without boiling [39] or (B) by fluorescence microplate reader. Each error bar represents the standard error of triplicate measurements. (C,D) The three strains were treated with serial concentrations of arsenic from 10 to 100 μg/L. (C) Fluorescence intensity of log-phase induced culture. (D) Cell lysates from full-growth cultures were visualized by fluorescence imager.

Fig. 3 Characterization of biosensor strain. (A) Dose and time dependent assay of biosensor strain. Single colony was picked to preculture followed by 1:25 fold dilution on the following day. Log phase induction was done with sodium arsenate 0 to 100 μg/L. Fluorescence was measured using 96 well microtiter plates at 1, 2 and 4 h respectively. (B) Lifetime determination of biosensor strain. Log-phase cells were kept at room temperature or 4°C in broth (liquid) or pellet state. On each day onward, 200 μL of stored cultures were incubated 96 well plates and induced with 10 μg/L arsenic. Fluorescence was measured after 3 h incubation. All incubation was performed at 37°C. (C) Specificity test of arsenite-responsive bacterial biosensor. Metals other than arsenic were used in 5 mg/L while arsenic was used in 50 μg/L. Fluorescence levels were measured after induction with different elements for 3 h at 37°C. Each error bar represents the standard error of triplicate measurements.

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Fig. 4 The scheme of novel dual selection system. (A) ON (CPR) selection of ArsR mutants in the presence of As(III). A pET-*arsR* variant plasmid library is created by error-prone PCR and cloning. The library is transformed into *E. coli* with pACYC-*Tth*. The DNA polymerase expression repressed by ArsR is restored by As(III). When each cell is compartmentalized by water-in-oil emulsion and subjected to emulsion PCR, heat lysis releases *Tth* pol leading to the amplification of the active variant genes. At this time, an *arsR*(high) gene with high sensitivity against As(III) is expected to be amplified more than an *arsR*(low) gene with low sensitivity. However, inactive variants (with stop codon or frameshift mutations) also resulted in *Tth* pol production in *E. coli* and are designated as false positive (FP). (B) OFF selection of ArsR mutants in the absence of As(III) to remove inactive FP variants. The *arsR* variant genes recovered from emulsion PCR is re-cloned into plasmid and is transformed into *E. coli* with pACYC-*sacB*. Because the SacB expression is lethal to *E. coli* in the presence of sucrose, the false positive variant lacks the ability to repress downstream gene expression leading to sucrose sensitivity in the absence of As(III). The active clones resistant to sucrose are taken for the next round of ON selection.

Fig. 5 Proof-of-principle experiment for CPR selection. (A) Expression and activity of DNA polymerase in *E. coli* containing dual plasmids pET-*arsR*/pACYC-*Tth* in the presence of 10 μg/L of As(III) were confirmed by Western blotting (top) and PCR (bottom), respectively. (B) *E. coli* cells with pET-*gfp*/pACYC-*Tth* and pET-*arsR*/pACYC-*Tth* were mixed in a ratio of 1:10 and subjected to single round of CPR selection (emulsified). The resulted PCR products were analyzed by agarose gel electrophoresis.

Fig. 6 Proof-of-principle experiment for OFF selection. (A) Growth of *E. coli* with pET-*arsR*/pACYC-*sacB* induced with or without As(III) (50 μg/L) was examined on agar plates with or without sucrose. (B-D) *E. coli* cells having pET-*arsR*/pACYC-*sacB* and pET-*gfp*/pACYC-*sacB* were mixed in a ratio of 10:1 and then subjected to single round of OFF selection in the absence of As(III). The *arsR* or *gfp* gene on pET-vector in

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colonies grown on plates containing no sucrose (B; before selection) or 5% sucrose (C; after selection) were amplified by colony-direct PCR and analyzed by agarose gel electrophoresis. (D) The number of colonies containing pET-*gfp* (black bar) or pET-*arsR* (white bar) before and after OFF selection were counted.

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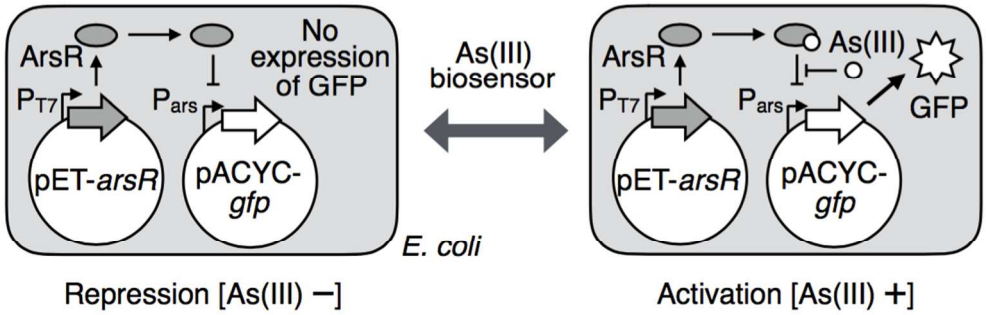

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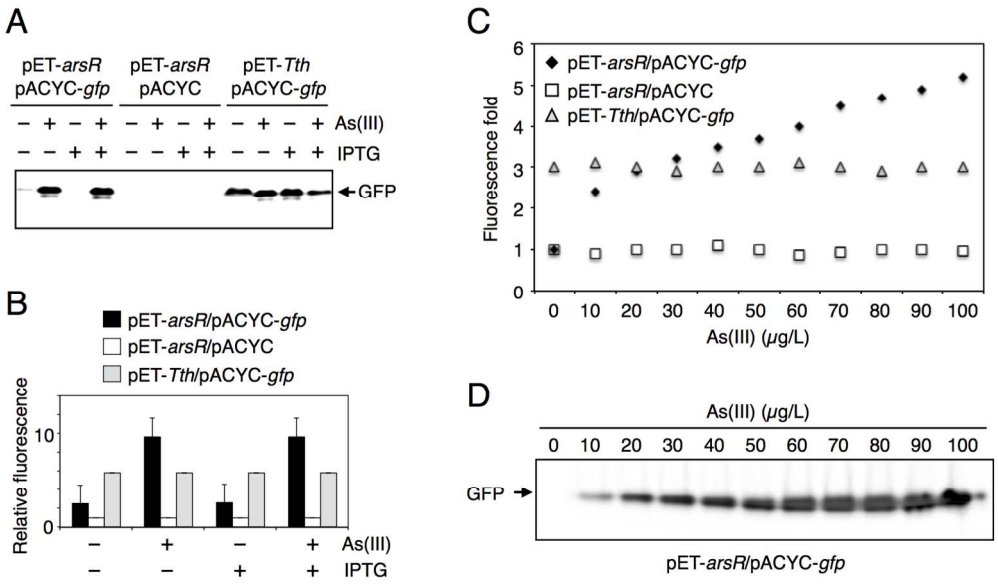

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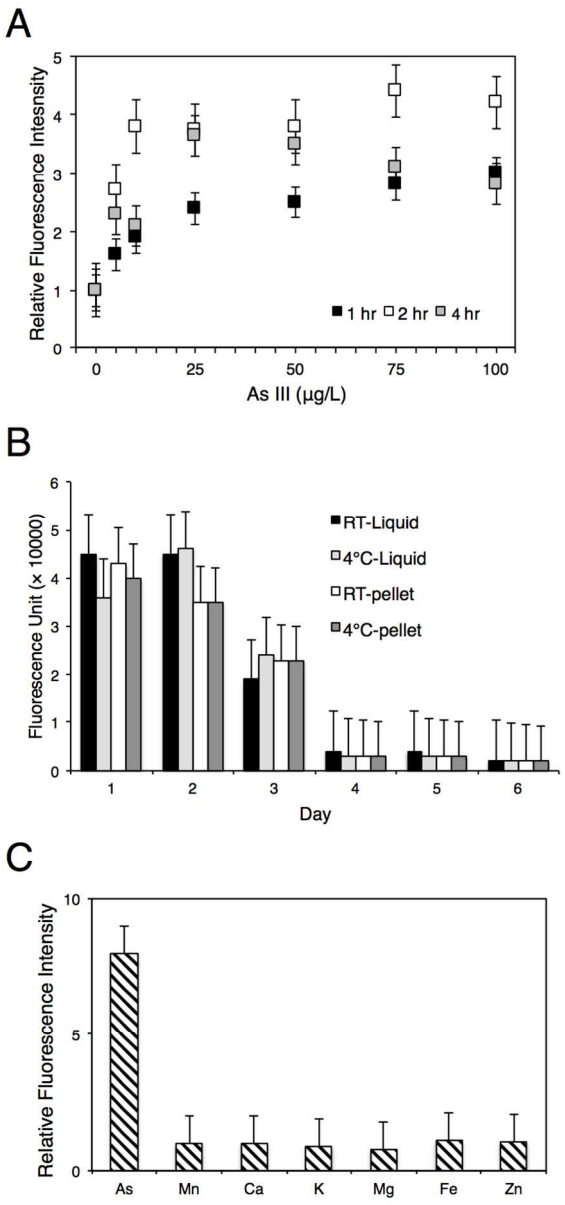

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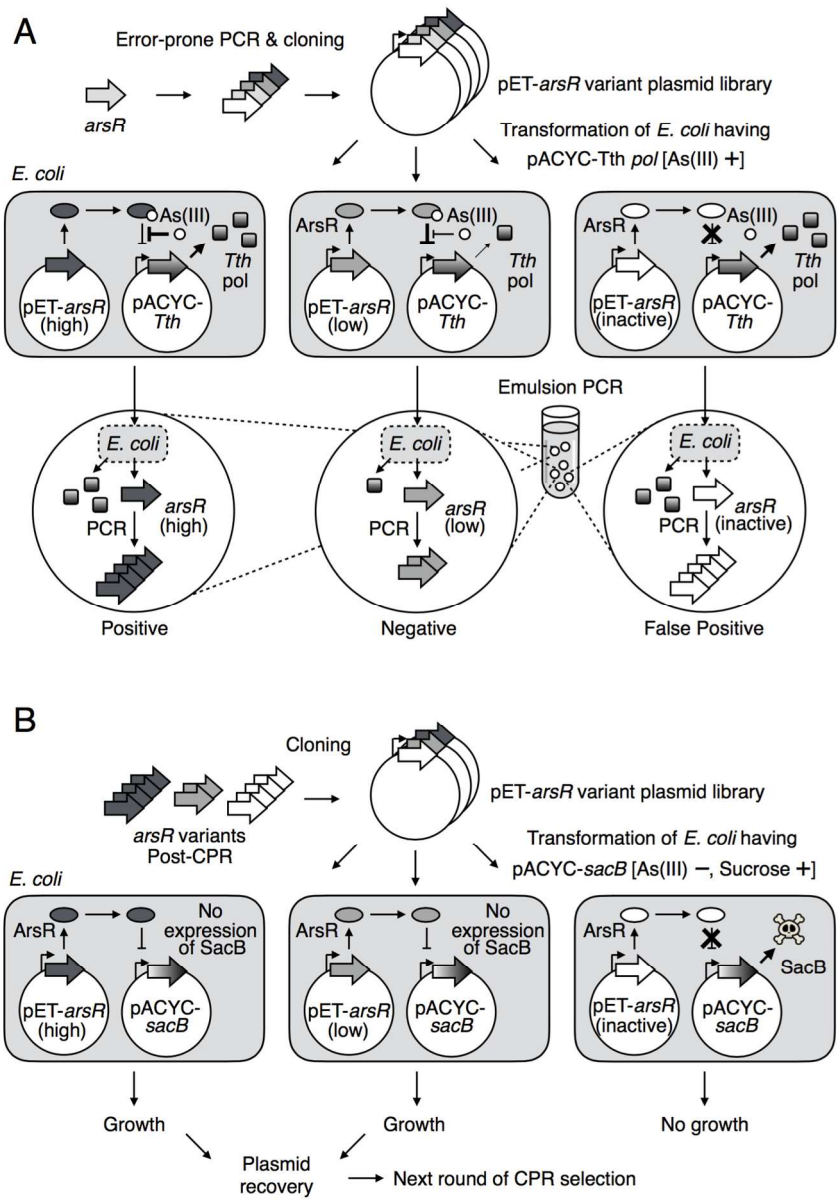

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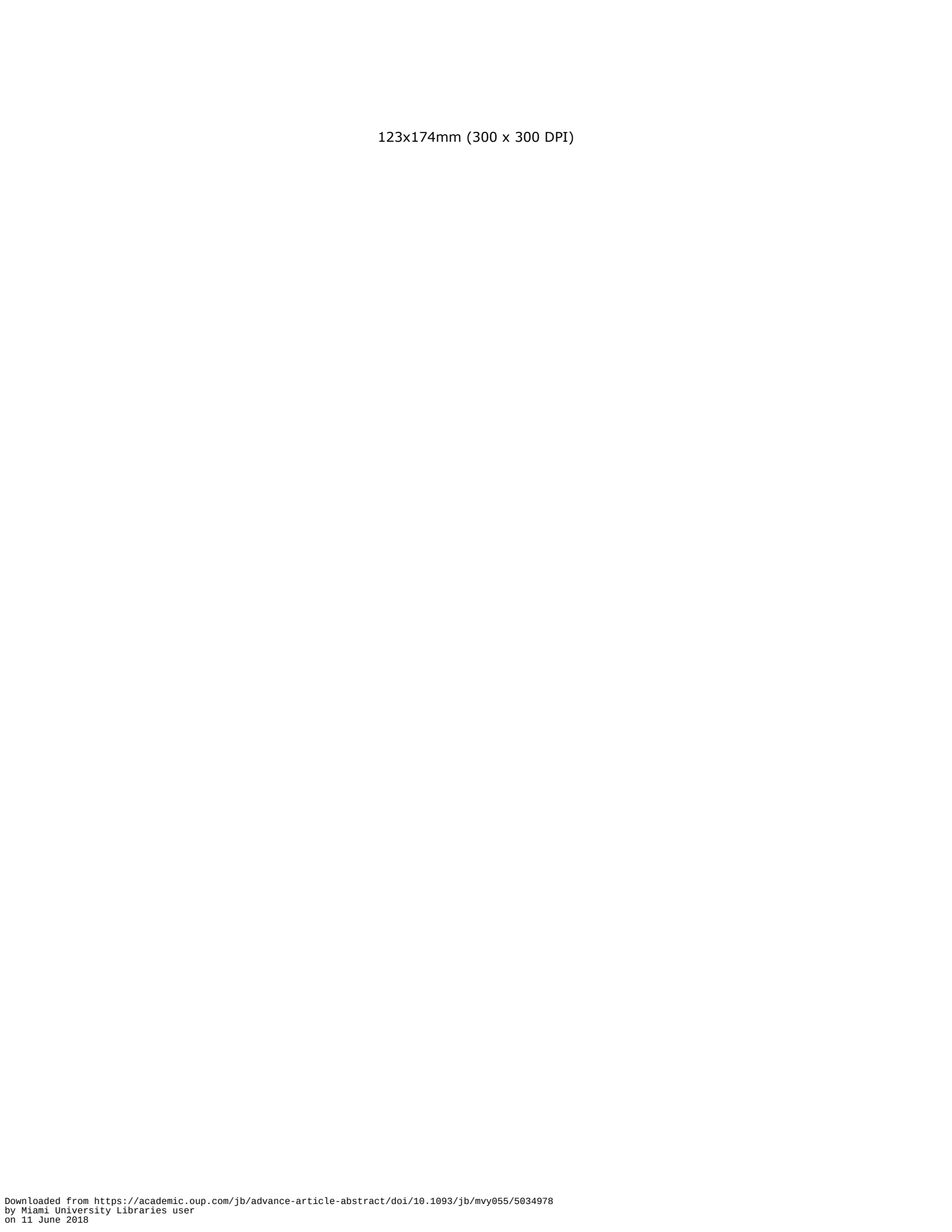

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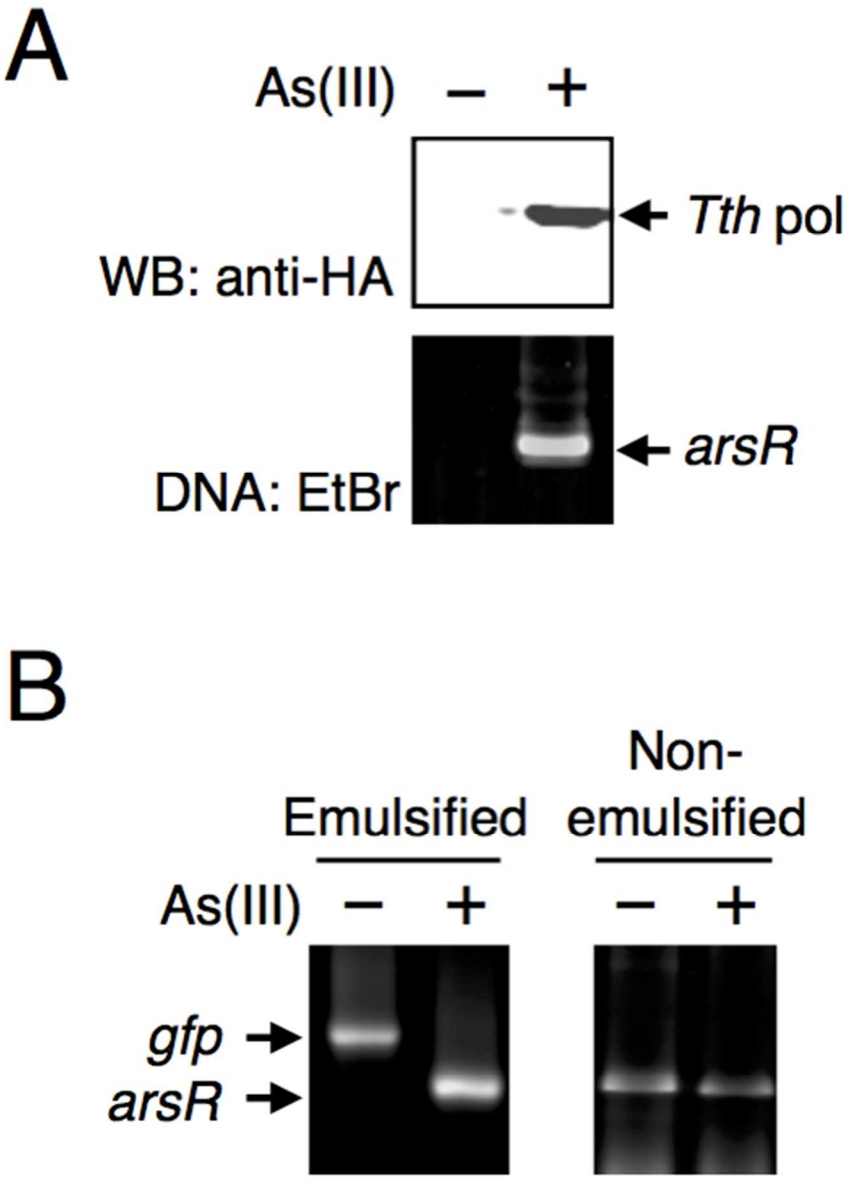

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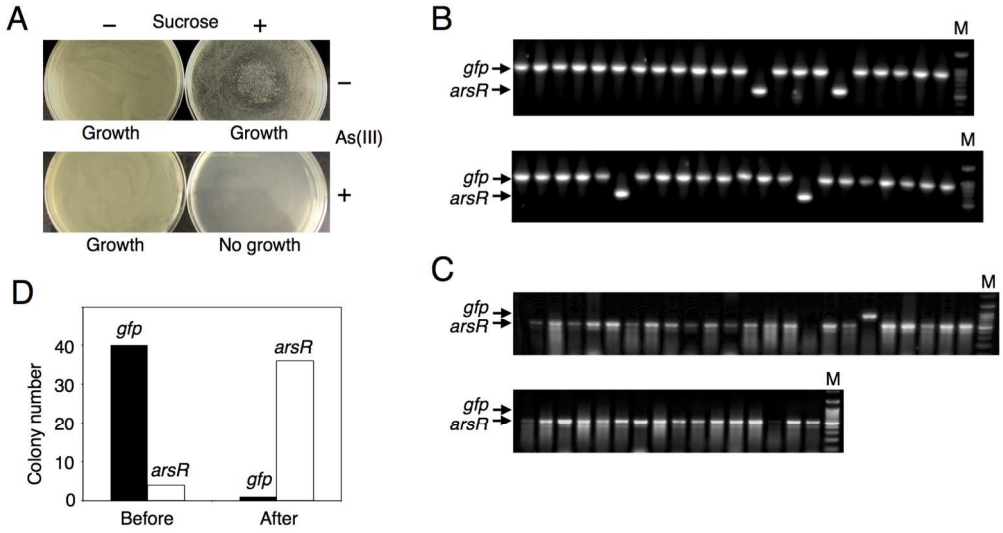

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