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Comparison of three genetically modified *Escherichia coli* biosensor strains for amperometric tetracycline measurement

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ABSTRACT

Three separate genetic strategies, based upon the induced expression of three different genes (*lacZ*, *selA* and *nuoA*) were tested to provide the SciTox assay with sensitive and specific detection of the antibiotic tetracycline (Tet). All three strategies relied on gene induction from the *Tn10 tetA* promoter. Both *lacZ* and *nuoA* biosensors responded specifically and sensitively to sub-inhibitory concentrations of Tet. However, the *selA*-based assay was not sensitive enough to detect Tet in the SciTox assay. The detection limits for Tet of the *lacZ* and *nuoA* biosensor strains were 0.11 μg ml^{-1} and 0.0026 μg ml^{-1}, respectively, and their linear ranges were 0.1–1 μg ml^{-1} and 0–0.01 μg ml^{-1}, respectively. While *lacZ* has previously been used as a reporter gene in an amperometric bioassay, *nuoA* is a novel and more sensitive reporter gene. This is the first report in which a respiratory gene was used as a reporter gene in an amperometric biosensor. The results indicate that this approach can produce a highly sensitive detection system. In order to test whether the new system could be used to detect other chemicals, the *nuoA* gene was re-engineered to be driven by the copper-inducible *copA* promoter. Using this strain, the SciTox assay was found to be able to specifically detect copper and silver ions.

1. Introduction

The SciTox toxicity assay is a commercially available, whole cell microbial assay that measures toxicity through inhibition of bacterial respiration (Tizzard et al., 2004) and is currently in commercial use to detect and quantify wastewater toxicity. It is a rapid catalytic microbial method in which the natural co-substrate, oxygen, is substituted by the mediator potassium ferricyanide (KFCIII) (Pasco et al., 2005). Electrons derived from oxidation of the substrate are released into the electron transport chain and ultimately to the external mediator KFCIII. This process leads to the accumulation of reduced mediator in solution. Transfer of electrons from the mediator to an electrode poised at a suitable voltage can generate a measurable current and is used to quantify the magnitude of respiration inhibition and indirectly the toxicity.

In its current configuration, the Scitox assay measures toxicity nonspecifically and therefore cannot distinguish between toxicants. In order to create a bio-assay that can identify and quantify specific toxicants at concentrations lower than inhibitory levels, the SciTox assay was re-engineered. Three separate strategies were devised to achieve this objective. They were all based on gene induction in response to a specific toxicant. The antibiotic tetracycline (Tet) and the *Tn10 tetA* promoter, which is de-repressed in response to the presence of Tet, were used as the model system.

In the first strategy, the quantity of metabolisable carbon available to the bacteria in the SciTox assay was manipulated by a method used previously in yeast (Lehmann et al., 2000; Tag et al., 2007). In this method, lactose is used as the sole carbon source for bacteria in the SciTox assay. *Escherichia coli* strains lacking the *lacZ* gene do not produce β-galactosidase and are incapable of utilizing lactose as a carbon source. By re-introducing the *lacZ* gene fused to the *tetA* promoter into these cells, metabolisable carbon is only available to the cells in the presence of Tet.

In the second strategy, the overall redox activity of the respiratory enzymes was manipulated. For this method, the *selA* gene, which encodes the enzyme selenocysteine synthase (Tormay et al., 1998), was fused to the *tetA* promoter and transformed into a *selA* knock-out *E. coli* mutant. The *selA* gene catalyzes the reaction of serine to selenocysteine conversion on Ser-tRNA^{Sec} using monoselenophosphate as the selenium donor (Allmang et al., 2009; Forchhammer and Bock, 1991; Forchhammer et al., 1991). Selenium atoms have similar properties to sulfur atoms. However, using Selenium instead of sulfur, the catalytic rate of seleno-proteins is significantly increased (Tormay et al., 1998).

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In the third strategy, a key respiration gene was placed under the control of the *tetA* promoter such that, in the presence of Tet, the gene was expressed and the cells' respiration rate increased. For this method, the *nuoA* gene, which encodes a protein that is directly involved in bacterial respiration, was used. The *nuoA-N* operon encodes the enzyme NADH dehydrogenase I, which catalyses the transfer of electrons from NADH into the start of the respiratory chain (Wackwitz et al., 1999), and functions in both anaerobic and aerobic conditions (Calhoun et al., 1993). The *nuoA* gene was fused to the *Tn10 tetA* promoter and then transformed into a *nuoA* knock-out *E. coli* mutant.

2. Materials and methods

Bacterial strains, plasmids and growth conditions.

The bacterial strains and plasmids used in this study are described in Table 1. All bacteria were *E. coli* strains cultured in shaking flask culture in LB medium at 200 rpm, 37 °C, and supplemented where appropriate, with: ampicillin (Amp) 100 μg ml^{-1}, kanamycin (Kan) 50 μg ml^{-1}, and Tet 10 μg ml^{-1}.

2.1. Construction of plasmids

(pSong8): The Multi-cloning site (MCS) of pBluescript was replaced with a SalI restriction enzyme site by Polymerase Chain Reaction (PCR) using primers 5′-ATAG **GTCGAC** CAG CTT TTG TTC CCT TTA GTG-3′ and 5′-ATGA **GTCGAC** CAA TTC GCC CTA TAG TGA GT-3′ followed by SalI digestion and re-ligation. The *Lac* promoter region upstream of *lacZ* gene was removed by PCR using primers 5′-ATAG **CTGCAG** ACT GCC CGC TTT CCA GTC GG-3′ and 5′-AGGAA **AAGCTT** ATG ACC ATG ATT ACG CCA AGC. The product was digested with *PstI* and *HindIII* and ligated to the *Tn10 TetA* promoter amplified by PCR from *E. coli* genomic DNA using primers 5′-ATCAC **CTGCAG** AAT GGG AAT TGA CGT TCC TTC-3′ and 5′-AATCA **AAGCTT** TT TTC TCT ATC ACT GAT AGG GA-3′.

(pSong9): The *lacZ* gene region was removed from (pSong8) by PCR with primers 5′-AATCA **AAGCTT** TT TTC TCT ATC ACT GAT AGG GA-3′ and 5′-ATAG **GTCGAC** CAG CTT TTG TTC CCT TTA GTG-3′. The PCR product obtained was (pSong8) sequence lacking the *lacZ* gene and this product was digested with *HindIII* and *SalI* restriction enzymes and ligated to the *E. coli selA* gene amplified from *E. coli* genomic DNA by PCR using primers 5′-ATCA **AAGCTT** ATG ACA ACC GAA ACG CGT TC-3′ and 5′-ATAG **GTCGAC** CAG AGA TAT CGC GCA ATA CC-3′ also digested with *HindIII* and *SalI* restriction enzymes.

(pSong10): The *lacZ* gene region was removed from (pSong8) by PCR with primers 5′-AATCA **AAGCTT** TT TTC TCT ATC ACT GAT AGG GA-3′ and 5′-ATAG **GTCGAC** CAG CTT TTG TTC CCT TTA GTG-3′ as described above. The PCR product was digested by *HindIII* and *SalI* restriction enzymes and ligated to the *E. coli nuoA* gene amplified from *E. coli* genomic DNA by PCR using primers 5′-ATCA **AAGCTT** ATG AGT ATG TCA ACA TCC ACT-3′ and 5′-ATAG **GTCGAC** TCA ACC GGG ATG AAT TTA TCG-3′ also digested with *HindIII* and *SalI* restriction enzymes.

(pSong11): The *tetA* promoter region was removed from (pSong10) by PCR with primers 5′-ATAG **CTGCAG** ACT GCC CGC TTT CCA GTC GG-3′ and 5′-AATCA **AAGCTT** TT TTC TCT ATC ACT GAT AGG GA-3′ and the products were digested with *PstI* and *HindIII*, followed by ligation with copA promoter amplified by PCR from *E. coli* genomic DNA using primers 5′-ATCAC **CTGCAG** ATT TTG TCC GCC GTT AAG TG-3′ and 5′-TGAC **AAGCTT** CAC TCC TTT AAG ACA GTT TTG ACT-3′.

2.2. PCR and DNA sequencing

Polymerase chain reaction (PCR) for construction of plasmids was by High Fidelity DNA polymerase (Invitrogen) as per manufacturer's instructions. The PCR reactions were: 94 °C for 3 min followed by 35 cycles of: 94 °C for 30 s; 60 °C for 30 s and 72 °C for 1 min followed by one step at 72 °C for 7 min. All plasmids were verified by sequencing the insert and bordering sequences. PCR-amplified DNA fragments were purified with PCR purification kit (Purelink^{TM}, Invitrogen, Carlsbad, USA), and quantified by nano-drop ND-1000 (100 W. Rockland RD, Montchanin, DE, USA), measuring the absorbance at 260 nm (A_{260}) and sequenced at the Bio-Protection Center hosted by Lincoln University using ABI Prism 3130x/Genetic analyzer.

2.3. Blue/white color screen (pSong8)

Top10f′ derivatives, with or without the specific plasmids, were grown at 37 °C for 16–20 h on LB agar plates, supplemented with ampicillin, Tet and 0.5 mM IPTG and 20 μg ml^{-1} X-gal.

2.4. Scitox assay

Two different methods were used for preparing cells for the SciTox assay. The method chosen for each strain was the method that worked best for that strain in initial experiments.

2.5. Method one

E. coli from freshly grown overnight cultures were harvested by centrifugation at 13 K rcf at room temperature. The cells were washed three times in phosphate buffer (KH_2PO_4 10.62 mg L^{-1}, K_2HPO_4 21.25 mg L^{-1}, KCl 7.46 mg L^{-1}). The cell concentration was adjusted to an optical density at 600 nm (OD_{600}) of 5, measured using an UNICAM 8625 UV/VIS spectrophotometer. The SciTox assay was performed in 24-well microtest plates. 1 ml of cells (or 925 μl of cells plus 75 μl of 252 mM lactose for *E. coli* (pSong8)), were incubated with 1 μl of variable concentrations of Tet at 37 °C, 200 rpm for 2 h; then 300 μl of 250 mM KFCIII was added to each well.

2.6. Method two

E. coli was grown until late-exponential growth phase (OD_{600} was around 0.5–0.8). The cells were not harvested by centrifugation but maintained in culture media. The SciTox assay was performed in 24-well microtest plates. 1 ml of cultured cells was incubated with 1 μl of variable concentrations of Tet. The plates were incubated at 37 °C, 200 rpm for 2 h; then 300 μl of 250 mM KFCIII was added to each well.

For both methods, oxygen was removed from the headspace by nitrogen flushing and excluded from the plates by sealing with an adhesive acetate lid. The microtest plates were wrapped in aluminum foil to exclude light, and incubated on an orbital shaker at 37 °C, 200 rpm for 1 h. Assays were terminated by transferring the contents of each well into 1 ml Eppendorf tube and centrifuging at 13 K rcf for 4 min to pellet cells. The supernatants were transferred into clean Eppendorf tubes. The samples were stored at 4 °C until microelectrode amperometry analysis could be performed.

2.7. Microelectrode amperometry signal detection

Limiting current microelectrode amperometry, 3-electrode system comprising a working, reference and auxiliary electrode, was used to measure the quantity of reduced mediator produced during the microbial incubation. The reference and auxiliary electrode were directly immersed in phosphate saline buffer supporting electrolyte, contained within a cell vial (BAS MF-1082). The 1 ml sample was confined in a separate working-electrode compartment (BAS MF-2031, 2 ml mini-cell). 200 μl samples were injected into BAS

Table 1
E. coli strains and plasmids.

Strain/plasmid	Genotype/characteristics	Source
BW25113	rrnB DElacZ4787 HsdR514 DE(araBAD)567 DE(rhaBAD)5 68 rph-1	Keio[a]
JW4364	BW25113 *ΔarcA*	Keio[a]
JW2283	BW25113 *ΔnuoA*	Keio[a]
JW3564	BW25113 *ΔselA*	Keio[a]
DH5α	endA1 hsdr17 supE44 thi-1 recA1 gyrA relA1Δ(lacZYA-argF) U169 deoR (phi 80lacZ Δ M15)	Invitrogen
Top10f′	F′{*lac*Iq Tn*10* (TetR)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
pBluescript	Vector encoding the N-terminal α-fragment of the β-galactosidase (*lacZ*) in frame with 21 multi-cloning sites; [Amp^r]; *lacZΔM15 lacI^q*	Stratagene
pSong3	pBluescript, with *lacZ* replaced by *selA* coding region	This study
pSong8	pBluescript with lacZ fused to the *Tn10 tetA* promoter	This study
pSong10	pBluescript with *lacZ* replaced by *nuoA* coding region fused to *tetA* promoter	This study
pSong11	pBluescript with *lacZ* replaced by *nuoA* coding region fused to *copA* promoter	This study

[a] Bacteria from the Keio collection have a kanamycin resistance cassette replacing the deleted gene. This cassette was not removed and all mutants are kanamycin resistan.

MF-2031, 2 ml minicell. The 25 μm platinum working-electrode was used at +450 mV relative to the Ag/AgCl reference electrode and anodic current obtained 10 s after imposition of the applied potential was taken as the limiting current value. After each analysis, the working-electrode was re-polished and the BAS MF-2031 minicell rinsed in distilled water and dried using lint-free tissue. The limiting measured current in microelectrode amperometry is proportional to the amount of KFCIII reduced during the 1 h incubation with the induced cells.

2.8. Experimental procedure

Firstly, three different sensors were exposed to a large-scale Tet concentration range, and the entire dose-response was profiled. Then attention was restricted to the dose-dependent response range and the experiments were repeated in order to find out the linear response range of the sensors. Biosensor strains were regularly maintained for long term storage in a −80 °C freezer. Cells were streaked onto LB plates supplemented with appropriate antibiotics, incubated overnight at 37 °C and stored for up to 2 weeks at 4 °C. For experiments, individual colonies from plates were grown in flask culture overnight and used in experiments the next day. No issues of strain instability were detected.

3. Results and discussion

3.1. Assay based on β-galactosidase expression

In order to convert the SciTox assay into a specific and more sensitive sensor, the *E. coli* biocomponent was re-engineered for Tet-induced expression of proteins that were expected to modify the SciTox response. In the first strategy, *E. coli* was transformed with the plasmid pSong8. *E. coli* (pSong8) expresses β-galactosidase in response to Tet. The expression of β-galactosidase allowed the

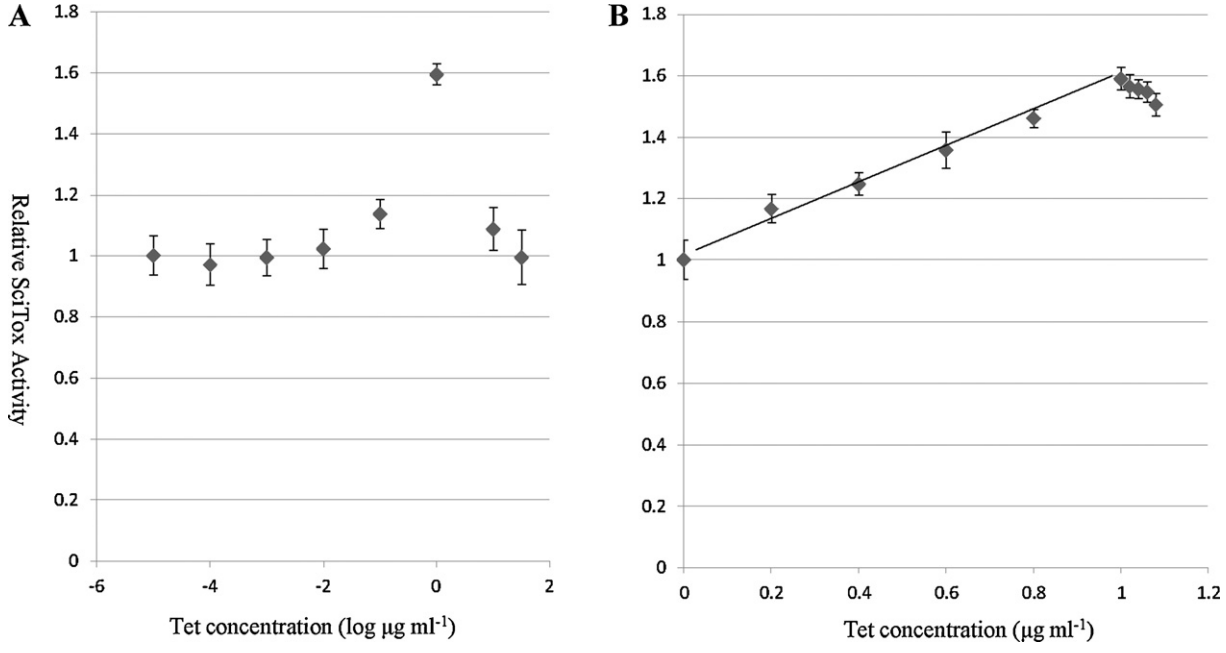

Fig. 1. Scitox response (mean ± SD) of *E. coli* (pSong8), exposed to Tet in water sample. (A) Broad concentration range. (B) Dose-dependent range. The fitted linear regression line is $y = 0.56x + 1.02$, $R^2 = 0.99$ for the dose-dependent data range. Tet concentration in (A) is in log scale and in (B) is in linear scale.

cells to metabolize X-gal and produce a visible blue color. Two different growth phases were studied by blue-color assay, based on the development period of blue color measured by spectrometer (data not shown). There was no measurable blue color developed by cells of both growth phases within 300 min, in the absence of Tet. However, in the presence of Tet the stationary-growth phase sensors developed blue color much faster than cells grown to exponential phase. This result indicated that, for the pSong8 biosensor, the stationary phase cells were more responsive to Tet.

The β-galactosidase expression allowed the cells to metabolize lactose and convert it into galactose and glucose. Glucose has been used as a substrate to increase SciTox signal in previous studies (Pasco et al., 2005; Tizzard et al., 2004). Based on this, *E. coli* (pSong8) was employed to detect Tet in the SciTox system. The SciTox response to increased concentrations of tet detected by *E. coli* (pSong8) could be described as: at very low concentrations of tet, there was no dose-dependent response detected; at higher levels there was a dose-dependent increase in SciTox signal as expression of β-galactosidase was induced; the signal increased to a maximum signal after which there was a dose-dependent decline in the signal at higher Tet concentrations (Fig. 1). The assay was approximately an order of magnitude more sensitive than the unmodified Scitox assay in response to Tet (Fig. 1). The limit of detection was 0.11 μg ml^{-1} and the linear dose-response range was 0.1–1 μg ml^{-1} (Fig. 1). At Tet concentrations above 1 μg ml^{-1}, the SciTox response declined. This was not due to antibiotic toxicity as the *E. coli* TOP10f′ cells are Tet resistant. The same results were observed in the white-blue color screen, 90% of colonies were white and light-blue color, when Tet concentrations were over 1 μg ml^{-1}. Loss of β-galactosidase activity may be due to over-expression of the transgene leading to the formation of inclusion bodies (Sambrook and Russell, 2001).

3.2. Unmodified SciTox assay response to Tet

In the SciTox toxicity assay, unmodified *E. coli* K-12 acts as the biorecognition element in the whole-cell based bioassay. Toxicity, including inhibition by antibiotics, should non-specifically inhibit *E. coli* respiration, resulting in lower SciTox signals. This result is seen in tetracycline sensitive strains exposed to inhibitory concentrations of tetracycline. However, at lower levels of some toxins, including tetracycline, an increase in signal was sometimes seen. The assay responded to Tet above 1 μg ml^{-1} (Fig. 2), with a detection limit of 4.11 mg ml^{-1}. The reason the unmodified *E. coli* assay responds to sub-inhibitory levels of some toxins is not known. The detection limit of unmodified assay was around 40 times higher than pSong8 assay.

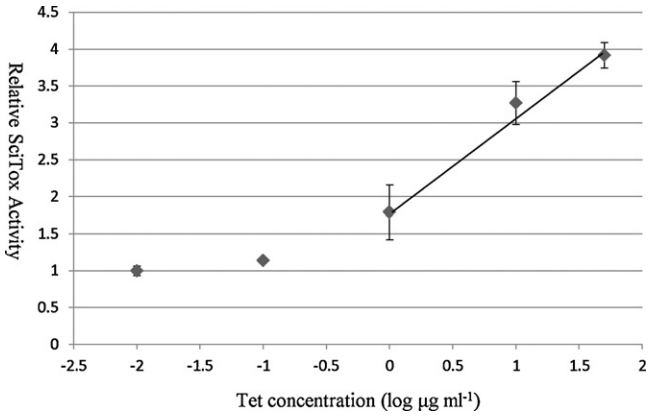

Fig. 2. Scitox response (mean ± SD) of buffer based unmodified *E. coli* K-12, exposed to Tet concentrations in water sample. The fitted linear regression line is $y = 1.27x + 1.85$, $R^2 = 0.99$ for the dose-dependent data range. The tet concentration is in log scale.

3.3. Assay based on selA expression

In *E. coli* (pSong9), expression of the *selA* gene, encoding selenocysteine synthase, is induced in response to Tet. Expression of *selA* increases the activity of seleno-proteins including some proteins with redox activity. However, the SciTox assay using *E. coli* (pSong9) as the biocomponent had a similar response profile to the assay using unmodified *E. coli*. The limit of detection for this assay was 1.45 μg ml^{-1} Tet (Fig. 3), which is not significantly different to the

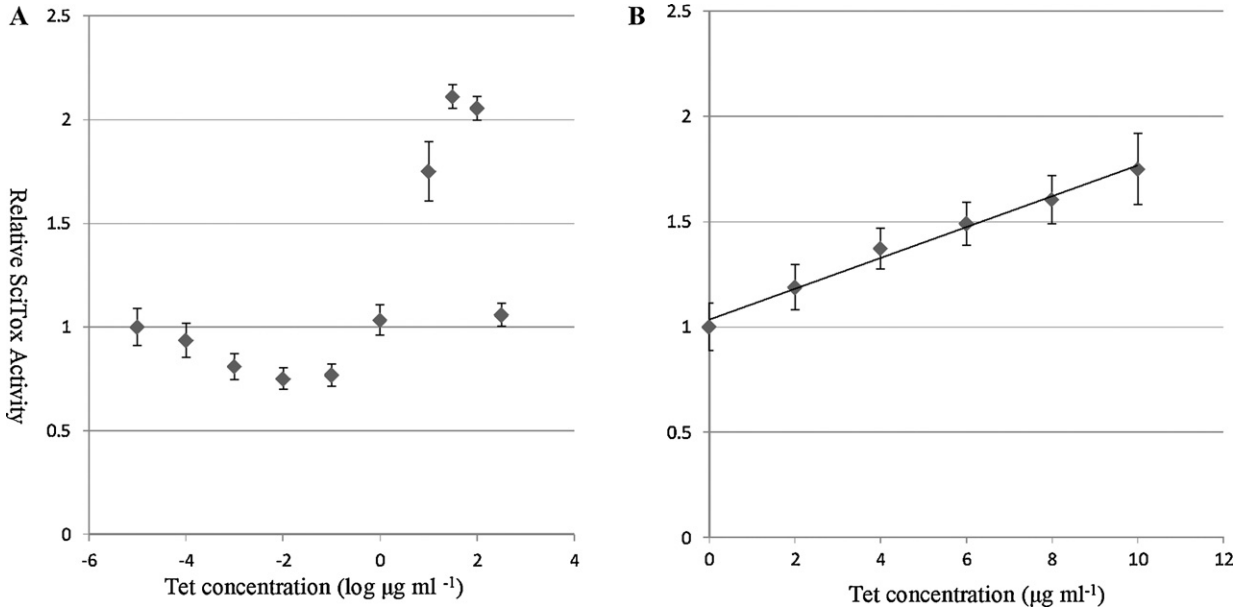

Fig. 3. Scitox response (mean ± SD) of *E. coli* (pSong9) sensor in stationary phase, exposed to Tet concentrations in water sample. (A) Broad concentration range. (B) Dose-dependent range. The fitted linear regression line is $y = 0.07x + 1.04$, $R^2 = 0.99$ for the dose-dependent data range. Tet concentration in (A) is in log scale and in (B) is in linear scale.

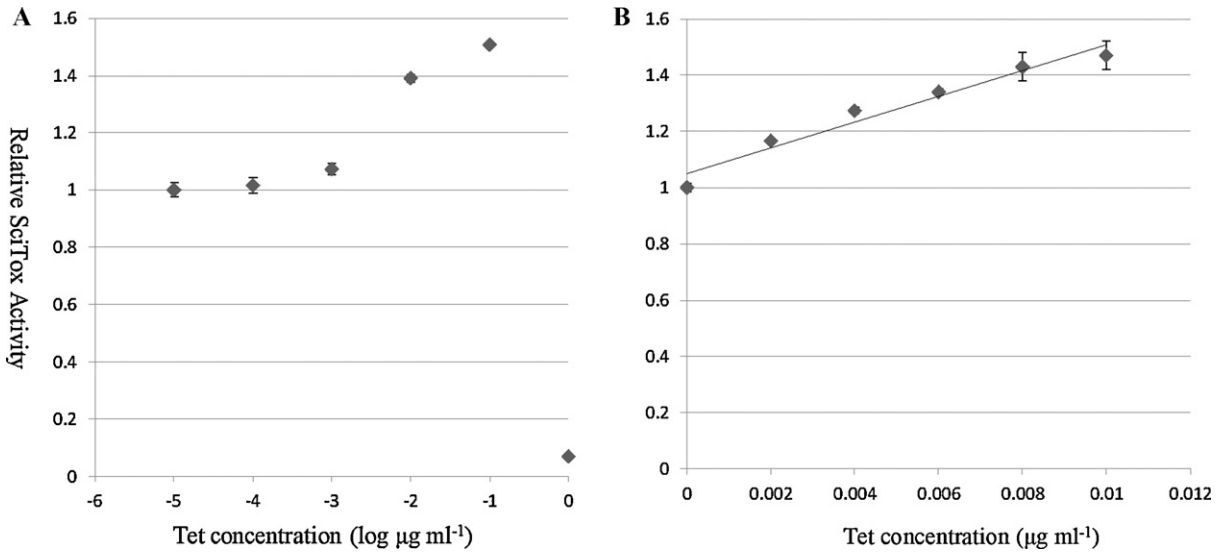

Fig. 4. Scitox response (mean ± SD) of sensor *nuoA*⁻ (pSong10), exposed to different Tet concentrations in water. (A) Broad concentration range. (B) Dose-dependent range. The fitted linear regression line is $y = 45.75x + 1.05$, $R^2 = 0.96$ for the dose-dependent data range. Tet concentration in (A) is in log scale and in (B) is in linear scale.

limit of detection for the unmodified SciTox assay. The linear dose-response range to Tet was from 0 to 10 μg ml^{-1} (Fig. 3).

The result might indicate that the response to Tet was mainly the non-specific response of the *E. coli* host rather than tet-induced reporter gene expression. It is not known why the *E. coli* (pSong9) assay did not work. It is not directly the action of SelA protein but the action of the newly synthesized seleno-proteins after the induction of *selA* that increases respiration rate. We speculated that the *E. coli* (pSong9) assay might work better if the cells were at exponential phase rather than stationary phase. However, when the *E. coli* (pSong9) assay was repeated using cells harvested at exponential phase the results did not improve (data not shown). It is unclear why *selA* cannot be used as a reporter gene to detect Tet sensitively in SciTox system, as *selA* expression is known to significantly affect the SciTox response (Weld et al., 2010).

3.4. Assay based on nuoA expression

In *E. coli* (pSong10), the *nuoA* gene is fused to the *tetA* promoter on the plasmid pSong10, carried in a *nuoA* deletion strain. The *nuoA* gene, encoding a subunit of the respiratory enzyme NADH dehydrogenase I, was expressed in response to Tet in this assay. The *nuoA-N* operon encodes the proton-pumping NADH dehydrogenase I which is composed of 13 protein subunits encoded by genes *nuoA-N* (Friedrich et al., 1995; Weidner et al., 1993). NADH dehydrogenase I transfers electrons from NADH into the start of the respiration chain and loss of *nuoA* gene expression has been shown to impair the Scitox response (Weld et al., 2010).

The assay using *E. coli* (pSong10) did not respond in a dose-dependent manner to Tet (data not shown). All the assays described here used cells that were grown overnight and harvested in stationary phase. Stationary phase cells were used as these were effective and convenient for use in the SciTox assay. Also, previous comparisons between stationary phase and exponential phase cells in the assay using *E. coli* (pSong8) indicated that stationary phase cells gave a more sensitive response than exponential phase cells (data not shown). However, *nuoA-N* expression is strongly growth phase-dependent; it is primarily expressed in early exponential phase and is significantly reduced after late exponential phase (Wackwitz et al., 1999; Zhang et al., 2004). It might be that, despite induction

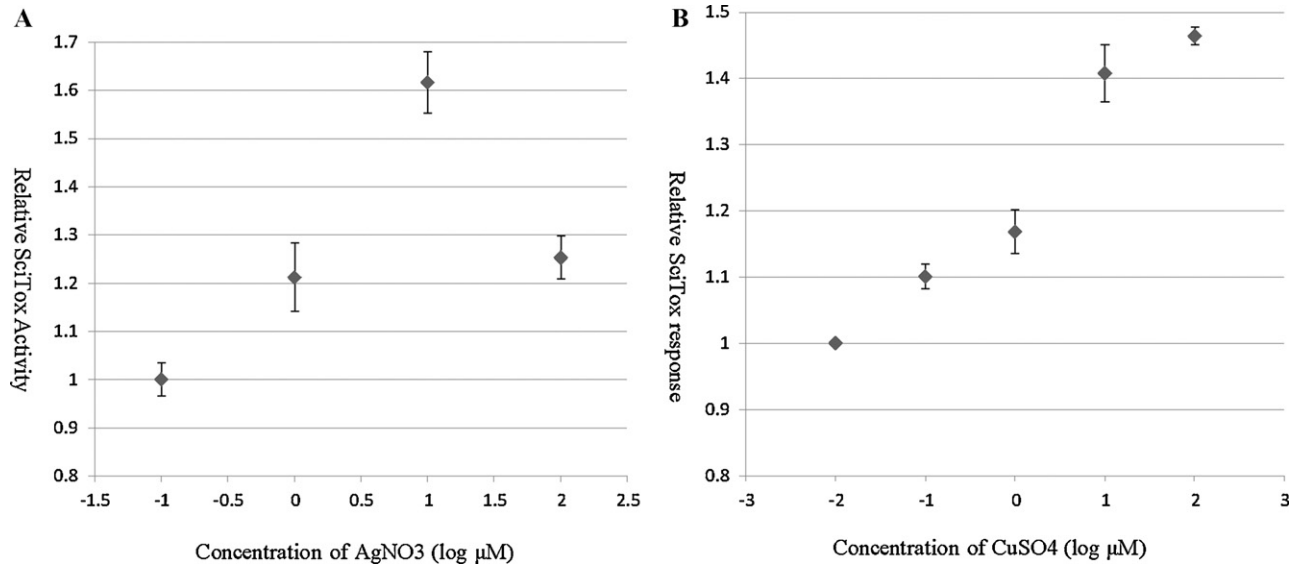

Fig. 5. Scitox response (mean ± SD) of sensor *E. coli* (pSong11), exposed to different concentrations of $AgNO_3$ (A) and $CuSO_4$ (B) in water. Tet concentration is in log scale.

of *nuoA*, the other proteins needed for NADH dehydrogenase I formation were relatively unavailable in that growth phase.

The *E. coli* (pSong10) assay was repeated using cells harvested at exponential phase and was found to have a sensitive, dose-dependent response to Tet. The limit of detection for this assay was 0.0026 $\mu g\,ml^{-1}$ Tet (Fig. 4), which is much more sensitive than the limit of detection for the *E. coli* (pSong8) SciTox assay. The linear dose-response range to Tet was from 0 to 0.01 $\mu g\,ml^{-1}$ (Fig. 4B).

The biosensor specificity to tetracycline and selectivity in different matrix was also tested. No response to a range of concentrations of the antibiotics ampicillin and kanamycin was detected and the biosensor was able to detect tetracycline in milk.

3.5. The nuoA reporter assay based copper and silver detection

In order to confirm that the *nuoA* reporter gene could be used to detect other, non toxic chemicals, using other inducible promoters, the *copA* promoter (specifically induced by Cu, Au and Ag ions) (Stoyanov et al., 2001) was fused to the *nuoA* reporter gene in pSong11. *E. coli* pSong11 specifically responded in a dose-dependent manner to both Cu and Ag in the SciTox assay (Fig. 5). This result confirmed that *nuoA* could act as a respiratory reporter gene for an amperometric sensor to specifically and sensitively detect targets in the SciTox assay.

3.6. Unmodified SciTox response to Cu and Ag

In order to confirm that *E. coli* (pSong11) was responding specifically to copper and silver ions, unmodified *E. coli* was tested as the biorecognition element in the whole-cell based bioassay induced either by 10 μM $CuSO_4$, 10 μM $AgNO_3$ or not induced. There was no non-specific response. Also, the SciTox response of *E. coli* (pSong11) was measured when exposed to the metal cation Mg^{2+} with two different concentrations of $MgSO_4$. In the assay there was no significant difference between different $MgSO_4$ concentrations, confirming the specificity of the assays.

4. Conclusions

Three new SciTox biosensor strains were tested for their ability to quantify Tet. Two strains (*E. coli* (pSong8) and *E. coli* (pSong10)) responded specifically to Tet in a dose-dependent manner. The assays based on these 2 strains were considerably more sensitive than the unmodified SciTox assay and other similar DTA assays (Ertl et al., 2000) and may provide a strategy that could allow the SciTox assay to be developed as a sensitive and specific biosensor. However, the pSong9-based assay was not sensitive as an amperometric sensor to detect Tet in the SciTox system as it had a similar dose-response to the unmodified *E. coli* K-12 assay. The assay based on induced expression of *nuoA* was only able to detect the target analyte (Tet, copper and silver ions) when cells harvested at exponential phase were used, whereas the assay using induced expression of *lacZ* performed best using cells harvested at early-stationary phase. Tet-induced expression of *nuoA* was found to be the most sensitive assay by nearly two orders of magnitude. This assay is at least as sensitive as other whole cell biosensors reported to detect Tet using bioluminescence or fluorescence reporter genes (Bahl et al., 2005; Pellegrini et al., 2004; Scaria et al., 2009; Virolainen et al., 2008). To our knowledge, this is the first time this gene, or any other respiratory gene, has been used as the reporter gene for an amperometric biosensor. There is a possibility that the two assays (*lacZ* and *nuoA*) are complementary and could be used together to increase the range and accuracy of quantification.

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