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An engineered GFP fluorescent bacterial biosensor for detecting and quantifying silver and copper ions

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Abstract Presented here are two engineered bacterial biosensors for detecting and quantifying silver and copper ions. The biosensors contain a silver/copper resistance operon and a Green Fluorescent Protein gene that is strictly regulated through silver activated promoter regions normally found on a silver resistance gene (*sil operon*). The two biosensors efficiently detected silver and copper concentrations of 40 µM–300 µM and 20 µM–600 µM respectively. A strong correlation (R^2 = 0.90 or above) between silver/copper and GFP signal makes it possible to quantify the ions using a linear regression. At room temperature incubation, the GFP signal of the biosensors in Ag^+ saturated after 13 h. However, a detectable GFP signal was seen in 4 h.

Keywords Biosensors · Two-component systems · Silver · Copper · Environmental monitoring

Introduction

Ionic silver (Ag^+) is an effective antimicrobial agent widely used in the cleaning and medical industries (Silver 2003; Randall et al. 2015). However, this heavy metal has burdened the environment through its release in large quantities, and persistence as hazardous waste, which negatively affects waterways, microbial communities, plants and animals. (Gorsuch and Klaine 1998; Marambio-Jones and Hoek 2010; Yu et al. 2013).

There have been several efforts to develop methods for detecting and quantifying silver ions within aqueous samples. One analytical tool is induced coupled plasma spectroscopy (ICPS), which provides a fast and accurate detection and quantification of silver ions and other heavy metals (Allabashi et al. 2009). Graphene based chemical sensors have also been shown to be a specific, rapid, and sensitive technique for silver ion and heavy-metal detection (Varghese et al. 2015). Wen et al. (2010) developed a

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fluorescent-based protocol for silver ion detection by utilizing graphene oxide and silver-specific oligonucleotides. However, limitations to these techniques make them less effective for certain applications. ICPS lacks the ability to selectively quantify bioavailable silver ions, which are of environmental concern, rather than ions which have precipitated or have bonded to other chemicals (Rosen and Hieftje 2004; Olaniran et al. 2013). This analytical method is also not cost-effective or portable for in-field environmental monitoring (Anabitarte et al. 2012). Graphene sensors are highly sensitive at lower silver concentrations but lack the ability to quantify higher concentrations. Kaewanan et al. (2017) described a graphene-based silver detection protocol with an upper quantification limit of 0.6 μM.

Whole-cell biosensors have been used for a variety of applications and are able to only quantify bioavailable ions, which are more environmentally toxic (Pueyo et al. 2001; Buccolieri et al. 2010; Gui et al. 2017). Biosensors are relatively cheap, and are potentially more practical for in field applications (Justino et al. 2017). Several bacterial based biosensor systems have been engineered for detecting various metal pollutants (Cuero et al. 2007). For example, Ravikumar et al. (2012) developed a bacterial biosensor with a detection limit of 16 μM Zn^{2+} and 26 μM Cu^{2+} by utilizing CusRS and ZraRS two-component systems and a reporter gene, and have suggested a similar approach for detection of silver. However, as of now, a silver specific bacterial biosensor has not been developed.

Whole-cell biosensors are engineered by constructing a plasmid vector where a regulated promoter drives expression of a reporter gene, where effective promoters are found in bacteria which are able to survive under conditions of extreme heavy metals contamination (Rensing and Maier 2003). The persistent exposure to silver has led to the development of a Ag(I)-resistant pathogenic *Salmonella,* from which plasmid pMG101 was isolated (Phung et al. 2001; Silver 2003; Randall et al. 2015). The genetics of the resistance mechanism (*sil* operon), comprises of nine ORFs, two of which (*silR* and *silS*) encode a two-component silver sensor and regulator unit (Phung et al. 2001). This operon has been shown to convey resistance to 0.6 mM $AgNO_3$ and is thereby a potentially effective mechanism to develop a biosensor (Cioffi 2014). Ag^+ and Cu^{2+} ions are known to upregulate the gene expression of *cus* copper resistance mechanism through a similar CusRS system (Gudipaty et al. 2012). Hence, it is possible that the silver resistance regulation system may also be responsive to Cu^{2+} ions as well.

Presented here are two GFP fluorescence based bacterial biosensors for silver and copper ions. The two-component silver sensor and regulator detection mechanism (SilRS) from the *sil* operon activates a promoter region that is coupled to green fluorescent protein expression, creating biosensor plasmids pRADEK.1 and pRADEK.2. Both plasmids allowed for the detection of silver through GFP fluorescence, making it a potential tool for monitoring silver ions.

Results

Constructed biosensors are responsive to Ag^+ and Cu^{2+} ions, but not Zn^{2+} ions

Biosensing plasmids were constructed by fusing a *mutGFP* gene (see "Materials and methods") with silver regulated promoters from pMG101. Two biosensing plasmids were made, pRADEK.1 (containing *silE* promoter) and pRADEK.2 (containing *silABC* promoter). These constructs were then transformed into *E. coli* J53(pMG101) to create the bacterial biosensor. Both biosensors, RADEK.1 and RADEK.2 in LB broth without NaCl, produced a GFP signal in silver concentrations ranging from 0.04 mM to 0.31 mM. When LB medium contained NaCl (10 g/L), the formation of the silver precipitate (AgCl) resulted in fewer bioavailable ions. Hence, the biosensor was able to detect 0.04 mM to 5 mM of Ag^+. Similar results were observed with $CuSO_4$. The biosensors produced a GFP signal between 0.02 mM and 0.6 mM of Cu^{2+}. With both silver and copper, the upper detection range was due to the toxicity of the biosensors in higher concentrations of the heavy metal cations. $ZnSO_4$ was tested, and no GFP signal was detected, showing specificity towards Ag^+ and Cu^{2+} ions.

Correlation between silver and copper concentration and GFP signal allows for the quantification of the ions

At higher concentrations of silver and copper ions, the optical density of the biosensor decreases (Figs. 1, 2). Although a signal was produced at these concentrations, it was not proportional to the silver concentration since the biosensor cells were reduced in numbers. In order to compensate for this, the GFP signal [F] was divided by the optical density signal (595 nm), to give a relative fluorescence signal per cell (normalized signal). The r-squared values of the normalized biosensor and the GFP biosensor signals were calculated and compared (Supplementary Table 1). Strong correlations (R^2 above 0.9) were exhibited by the biosensors for both silver and copper ions in both LB with NaCl and LB without NaCl. Normalization of the GFP signal allowed for a larger correlation range with r-squared values comparable to non-normalized signals, except for pRADEK.1 and pRADEK.2 in LB without NaCl with silver, which did not show an increase within the concentrations that were tested (refer to Supplementary Table 1). This result suggests that a standard curve can be used for quantifying silver and copper ions.

Biosensors require a minimum of 13 h at 25 °C for the GFP signal to saturate

The time required for a system to accurately detect and quantify is an important factor when determining its use and applicability. An assay was performed to find the minimal time required for the biosensor to produce a GFP signal in various silver concentrations. The two biosensors were placed in 0, 0.02 mM, 0.2 mM, and 0.4 mM $AgNO_3$, and GFP and OD (595 nm) were monitored for 18 h post inoculation. The biosensors were incubated at room temperature (25 °C) to mimic the temperature that would be experienced during on-field testing. The GFP signal was normalized to the optical density (Fig. 3). GFP signals were greater than the 0 mM control at each concentration except 0.02 mM, were observed after 4 h. However, the normalized signal of the biosensors only began to saturate at 13 h. All timepoints post 13 h were not statistically greater than one another.

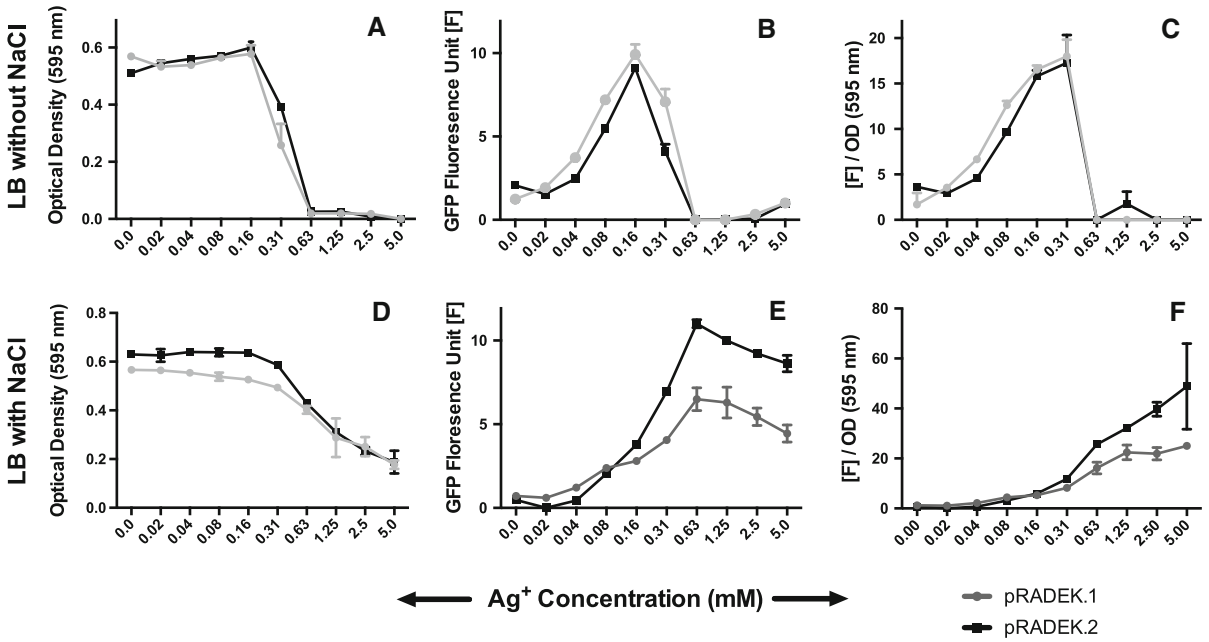

Fig. 1 Optical density (**a**, **d**), GFP signal (**b**, **e**) and normalized GFP signal (**c**, **f**) of pRADEK.1 and pRADEK.2 biosensor at Ag^+ ion concentrations in LB without NaCl (top row) and LB with NaCl (bottom row). When NaCl is present in the media, an AgCl precipitate forms resulting in different silver detection ranges (refer to **c** and **f**). The upper Ag^+ detection limit is caused by the biosensors becoming unviable in those concentrations

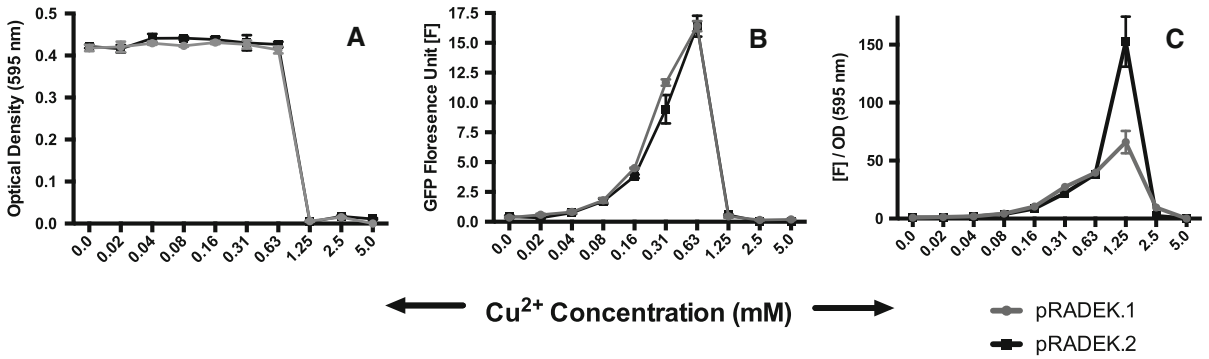

Fig. 2 Optical density (**a**), GFP signal (**b**) and normalized GFP Signal (**c**) of pRADEK.1 and pRADEK.2 biosensor at Cu^{2+} ion concentration in LB without sodium chloride. Biosensors are resistant and able to detect Cu^{2+} up to 1.25 mM. The upper Cu^{2+} detection limit is caused by the biosensors becoming unviable at those concentrations (refer to **a**)

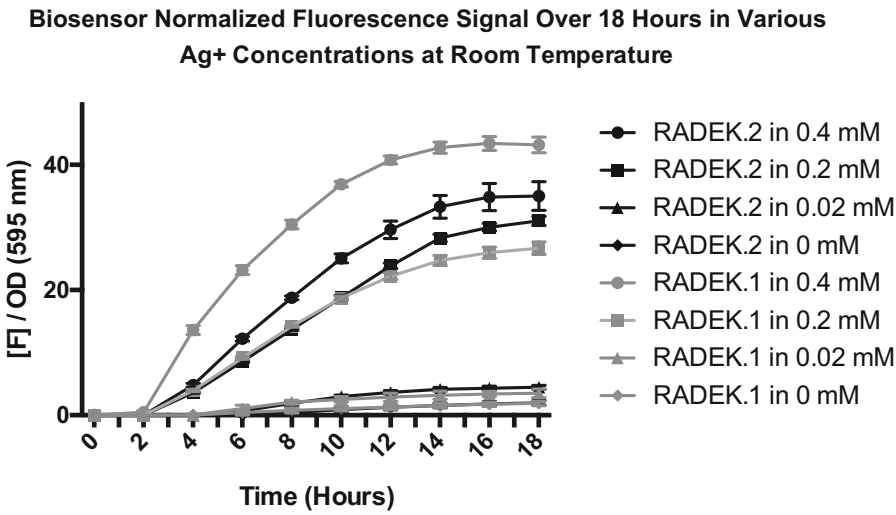

Fig. 3 Biosensors pRADEK.1 and pRADEK.2 normalized GFP signal in 0 mM, 0.02 mM, 0.2 mM, 0.4 mM Ag^{+} concentration over 18 h post inoculation. The signal saturated after 13 h. However, notable GFP signals are seen 4 h post-inoculation

Discussion

Both biosensors pRADEK.1 and pRADEK.2 detected Ag^{+} and Cu^{2+} concentrations. When the biosensors were exposed to Zn^{2+}, no GFP signal was detected showing that the biosensors are specific to silver and copper. At higher metal concentrations, the biosensor cells, although resistant, were reduced in numbers.

The presence of NaCl within the LB media affected the detection and quantification range of the biosensor for Ag^{+}. The presence of NaCl caused the formation of an AgCl precipitate that likely resulting in a reduction of bioavailable silver ions within the media. Since bioavailable ions are what the biosensor detects, the fluorescence signals of the biosensors in LB versus LB without NaCl are drastically different (Fig. 1). In addition, Ag^{+} ions are significantly more toxic to bacterial cells than silver chloride (Choi et al. 2008). This allowed for an increase in the detection and quantification range since the bacterial biosensor cells survive at higher concentrations. Since the biosensors were responsive to both Cu^{2+} and Ag^{+}, methods for making the biosensor specific to one metal, or to differentiate between the two metals through their signal is warranted.

Optimizing the time required for the biosensor to produce a signal is important for commercial applicability. Ravikumar et al. (2012) performed similar time optimization assays for a zinc and copper biosensor which utilizes a similar two-component signaling pathway. At an incubation temperature of 37 °C, a minimum of 8 h was reported for the GFP/RFP fluorescence signal to saturate. In the field, the assays described here determined the signal saturation point

at a temperature closer to possible in-field conditions. Although 13 h of incubation at room temperature is required for signal saturation, significant GFP signals at 0.2 mM and 0.4 mM were noticed after 4 h.

At lower concentrations, the GFP signal became less accurate likely due to noise from the GFP measurement method, and the low sensitivity of GFP as a reporter gene. For example, at 0.02 mM Ag^{+}, pRADEK.2 produced a GFP signal that was less than that produced at 0 mM. Issues with the low sensitivity of GFP as a reporter gene have been described before by Gui et al. (2017). Sensitivity of the biosensor at lower concentrations may be improved by using a more sensitive reporter gene such *luxABC*. GFP could possibly be substituted with more field practical reporters for visual screening such as *lacZ* and corresponding chromogenic or luminescent substrate.

There is no standardized protocol to implement optical microbial biosensors for environmental monitoring. Recent work suggests that biochips or lab-on-a-chip approach may work well because of their small size, easy operating procedure, and portability (Xu and Ying 2011). Horry et al. (2007) developed a portable optical biosensing device named Lumisens 2 that utilizes fluorescent microorganisms for detection of pollutants. It was effective and simple to use, making it well suited for field monitoring of environmental pollutants. Lee et al. (2007) used a cell array chip with twelve engineered bioluminescent bacteria to analyze oxidative stress. Adapting such technology for other microbial biosensors is one way of making them more accessible and applicable for on-field applications.

Materials and methods

Molecular cloning

Standard cloning protocols were used (Sambrook and Russell 2001). These methods include calcium chloride induced DNA transformations, restriction endonuclease digests and ligations, gel electrophoresis, PCR and gel extractions.

Microbial culturing and competent cells

Bacterial strains were either cultured in LB broth (with 10 g/L NaCl) or on LB plates with a selection antibiotic as appropriate (ampicillin 100 µg/ml or kanamycin 25 µg/ml). Transformation competent cells were made of both *E. coli* J53(pMG101), and *E. coli* DH5α using the method described by Sambrook and Russell (2001). These cells were stored at − 80 °C until use.

Plasmid and genomic DNA extractions and DNA quantification

Plasmid DNA was extracted using anion exchange columns after an alkaline lysis. Genomic DNA was extracted with standard techniques: Sodium dodecyl sulfate lysis, chloroform extraction, and ethanol precipitation. Final DNA products were suspended in a 50 µl solution, made of 0.2 M Tris at pH 8.0 (Tan and Yiap 2009). Plasmid DNA was quantified using Nanodrop 2000 (Thermo-Fisher Waltham MA USA).

PCR amplification of silver regulated promoters on pMG101

PCR primers were designed to amplify 244 and 245 bp of DNA before the transcription start point of both the *silE* (F: CAT TCT AGA AGT TGC ACT AAT TAT ATC GAA TGG CT, R: CAT GAA TTC TCC AGA CAA CGA AAA GGA GAA GG) and *silABC* (F: CAT TCT AGA GTG ATC CAC GAC GAA CCC C R: CAT GAA TTC AAA CGA AGT CCA GAG TAT TGA TAG GG) respectively, according to pMG101 sequence at Genbank accession number PRJNA20285. In addition, these primers contained EcoR1 and XbaI overhanging restriction endonuclease sites. Although the promoter may have been less than 245 bp, the exact location of DNA binding protein sites have not been characterized; 245 bp was safely large enough when comparing with defined promoters on other similarly regulated heavy metal resistance mechanisms like the *cus* operon. PCR amplification was done using the KOD amplification system (EMD Millipore), and a touchdown cycle program (Bio-Rad T100). Amplification was confirmed by running products on a 2% agarose gel stained with GelRed.

Engineering vector construct

pFPV25.1, containing a fluorescent *mutGFP* gene was purchased from Addgene (catalog number 20668). The plasmid contained an ampicillin resistance marker

which pMG101 happens to also convey resistance to. Thus, the fluorescent GFP gene from pFPV25.1 was cleaved using restriction enzymes (EcoR1 and HindIII) and ligated with EcoR1 HindIII cleaved plasmid pK19mobsacB (Schäfer et al. 1994). Ligation products were recovered by transformation of *E. coli* DH5α (Hanahan 1983), followed by selection on ampicillin and kanamycin. The insertion of the GFP gene into the plasmid pK19mobsacB was confirmed through restriction enzyme digestion and gel electrophoresis analysis. The newly constructed plasmid was designated pK19mobsacGFP.

Next, the promoter controlling the production of GFP in pK19mobsacGFP was replaced with the silver regulated promoter from plasmid pMG101. The existing promoter region from pK19mobsacGFP was removed and replaced with the PCR amplified promoter region (from *silE*, 244 bp in length, and from *silABC,* 245 bp in length) using restriction enzymes EcoRI and XbaI. The resulting two plasmids pRADEK.1 (containing *silE* promoter) and pRADEK.2 (containing *silABC* promoter) were obtained by transformation of *E. coli* DH5α. Successful promoter fusions were confirmed with digestion using the same restriction enzymes and gel analysis. pRADEK.1 and pRADEK.2 were then transformed into *E. coli* J53(pMG101) and selected using kanamycin and ampicillin. The resulting biosensor strains were subjected to further experimentation.

Biosensor sensitivity assay to silver, copper, zinc ions

The ability of the constructed biosensor strains to detect $AgNO_3$, $CuSO_4$ and $ZnSO_4$ was tested in a 96-well microtiter plate by using the 2-fold microdilution method with slight modifications. The biosensor was pre-cultivated in LB with 100 μg/ml ampicillin and 25 μg/ml kanamycin overnight at 37 °C (shaking 200 rpm), and then diluted to obtain an optical density reading of 0.2 at 595 nm. Ionic silver, and copper concentrations were tested at 10 mM, followed by twofold concentration dilutions until 0.02 mM. Control wells contained the culture in LB alone, LB and Ag^+ concentrations alone, and LB alone. After 24 h of incubation at 37 °C shaking at 200 rpm, the GFP light signal and optical density (595 nm) were measured. A Molecular Devices (Sunnyvale, California) FilterMax F5 was used to read standard clear well microplates. Before measurements were taken, the plate was shaken for 5 s. GFP fluorescence was measured using bandpass filters centered on 485 nm for excitation, and 535 nm for emission. This test was repeated for two types of LB media, one being the standard recipe, and second one being modified to contain no NaCl. NaCl can affect the availability of silver ions through the formation of a silver chloride precipitate. By removing NaCl from the LB media, a better measurement of the biosensors ability to detect free silver ions can be achieved. This test was repeated at various start concentrations to obtain data points in ranges that the biosensor was able to detect. In addition, the GFP signal for each concentration was subtracted from LB and silver with no cells to remove background noise. Data was examined for a positive correlation (linear correlation coefficient) between silver, and copper concentration and GFP signal. *E. coli* J53 (pMG101), *E. coli* J53 (pK19mobsacGFP), and silver sensitive *E. coli DH5α* were included as controls.

Biosensor GFP signal time optimization assay

The minimal time needed for the biosensor to produce a GFP signal at various [Ag^+] was determined by a time optimization assay. Biosensors *RADEK.1* and *RADEK.2* along with controls *E. coli* DH5α*, E coli* J53 (pMG101) and *E. coli* J53 (pMG101/pK19mobsacGFP) were cultured in LB without sodium chloride overnight at 37° with the appropriate antibiotics. The cultures were then subcultured into LB without NaCl with antibiotics until the optical density 595 nm reached approx. 0.5. The cultures were then induced with 0.4 mM, 0.2 mM 0.02 mM and 0 mM Ag^+ nitrate and incubated at room temperature (25°). At hourly timepoints between 0 h and 18 h the optical density 595 nm and GFP signal (485 nm and 535 nm) was recorded. Note that GFP and OD measurements were taken during different assays due to the inability for the spectrometer to automatically detect both during the same experiment. The GFP and OD at the beginning and end of each timepoint were compared and shown not to be statistically different between experiments, hence the GFP was divided by the optical density and plotted overtime.

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