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Constructed molecular sensor to enhance metal detection by bacterial ribosomal switch–ion channel protein interaction

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ABSTRACT

Molecular biosensors are useful tools that detect metal ions or other potentially toxic chemicals. However, the efficiency of conventional sensors is limited in mixed metals substrates, which is the common way they are found in nature. The use of biosensors constructed from genetically modified living microbial systems has the potential of providing sensitive detection systems for specific toxic targets. Consequently, our investigation was aimed at assembling different genetic building blocks to produce a focused microbial biosensor with the ability to detect specific metals. This objective was achieved by using a synthetic biology approach. Our genetic building blocks, including a synchronized ribosomal switch–iron ion channel, along with sequences of promoters, metal-binding proteins (Fe, Pb), ribosomal binding sites, yellow fluorescence reporter protein (YFRP), and terminators, were constructed within the same biobrick in *Escherichia coli*. We used an rpoS ribosomal switch containing an aptamer, which responds to the specific metal ligands, in synchronization with an iron ion channel, TonB. This switch significantly stimulates translation, as expressed by higher fluorescence, number of colonies, and concentration of RNA in *E. coli*. The positive results show the effectiveness of using genetically tailored synchronized ribosomal switch–ion channels to construct microbial biosensors to detect specific metals, as tested in iron solutions.

1. Introduction

Biosensors are useful cellular tools that allow the detection of a wide range of elements, in many cases overcoming the limitations of conventional methods. Although in nature metals are found in mixtures, multiple compounds, and ionized species, many conventional sensors fail to detect specific metals (Cuero et al., 2003; Cuero and Ouellett, 2005). Metal ions have been shown to be essential in the synthesis of nucleic acids (Failla, 1977) where the ligand binds with the nucleic acids (Cuero et al., 2003; Cuero and Ouellett, 2005). Through the introduction and coordination of multiple genes, biological systems have been designed to specifically identify target molecules, and these systems are termed biosensors (Prow et al., 2004). Such biosensors can be used to identify specific metals, which in nature are found in mixtures (Cuero et al., 2003; Cuero and Ouellett, 2005).

Heavy metals are an increasing pollutant of the environment and there is a growing need to develop sensitive and selective methods for their detection. Although metals enter the cell through different mechanisms, ion channels are common paths for metals to enter the cell. Voltage-gated ion channels are proteins embedded in the cellular membrane that allow the passage of ions into a cell through an electrochemical gradient and are target selective based on the tertiary structure of the protein (Hille, 2001). Different types of ion channels are found in bacterial cells; however, the voltage-gated type of ion channel seems to play important survival roles such as chemotaxis and homeostasis in prokaryotes (Ito et al., 2004). The voltage-gated Ton Box (TonB) ion channel allows the passage of bivalent cations, such as Fe (II), which was assembled for selective passage through the cellular membrane into the cell, oxidizing the ions as they enter. Some bacteria, such as *E. coli,* can exhibit natural protein receptors at the periplasm level, such as TonB, which could function as ion channels (Quintero et al., 2007). The Na^+ ion has been used in a voltage-gated channel in bacteria, showing a reciprocal modulatory effect on chemosensory transduction (Ito et al., 2004). The integration of ion channels into an artificial polymer membrane in combination with an optical sensor has been considered for drug screening and biochemical analysis (Steiner et al., 2003). An allosteric ribozyme has been engineered to respond to specific divalent metal ions (Zivarts et al., 2005).

Ribosomal switches are structured RNA domains found in many mRNAs of bacterial species that detect small molecules and control the expression of associated genes (Breaker, 2010). This

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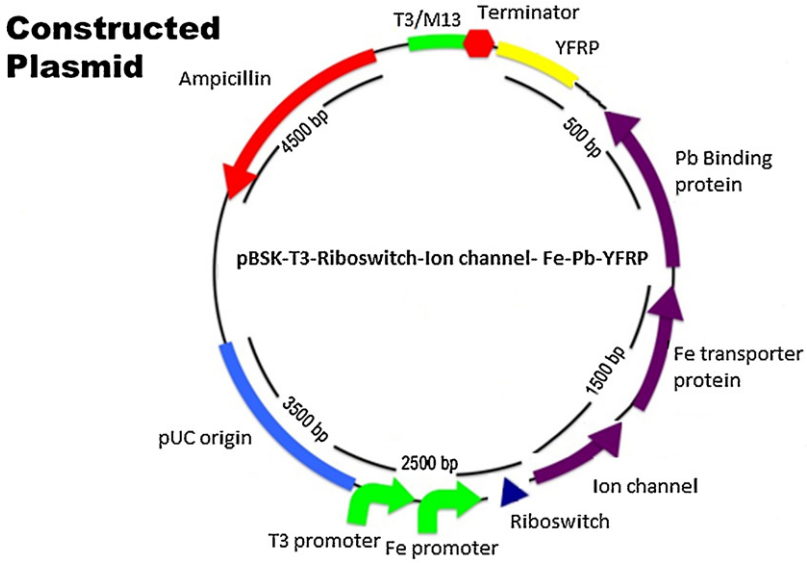

Fig. 1. Graphic illustration of constructed plasmid showing the direction, placement and size of genes within the pBSKII vector beginning with the T3 promoter, iron promoter, riboswitch rpoS, ion channel, iron-binding protein, lead-binding protein, yellow fluorescent reporter protein (YFRP), and the terminator sequence. Ampicillin resistance gene is included as a genetic marker and was used for screening colonies that had successfully been transformed with the plasmid.

function suggests that domain-ligand binding may form between RNA and metal ions. Riboswitches are a two-part system consisting of an aptamer, which changes conformation when bound to its metabolite, and an expression platform containing the regulated genes. The regulation of gene expression on a translational level can be mitigated by mRNA riboswitch folding that responds to small target molecules either activating or deactivating the translation of downstream genes (Nudler and Mironov, 2004). Selective riboswitches in response to environmental conditions in the presence of metal contamination can be used to control gene expression. The untranslated 5′ aptamer of the rpoS riboswitch binds to the stationary-phase sigma factor S1, which has been identified as a survival response to extreme environmental conditions, such as oxidative stress (Vitreschak et al., 2004). Based on such examples, the use of synchronized riboswitch–ion channels assembled with different gene building blocks has been used advantageously to detect specific metals (Quintero et al., 2007).

Synthetic biology is a developing scientific research approach that coordinates engineering and classical molecular genetics techniques to develop biological systems with specific functions (Andrianantoandro et al., 2006). This tool can be applied to the design, understanding, and operation of genetic systems through the assemblage of genes derived from a wide range of different organisms. Synthetic biology provides the ability to design biological systems with specific functions that do not exist in nature. It provides tools to engineer mechanisms for controlling genetic expression at the regulatory level, including detection of specific metals. The use of synthetic biology to develop biosensors for detecting Fe^{2+} was reported by Quintero et al. (2007).

A microbial biosensor is an analytical device that is able to accurately and sensitively detect specific targets in different fields, such as environmental monitoring, medicine, food processing, and food safety. Many of these microbial biosensors have been engineered based on the assemblage of synthetic genetic parts (Lei et al., 2006). However, despite the effective assemblage of the genetic parts or construction of the microbial biosensor, the metabolic activity is not good enough to express sensitive detection (Quintero et al., 2007).

In this study we apply concepts of synthetic biology in combination with conventional scientific methods to assemble different genetic components to construct a bacterial molecular biosensor for enhanced detection of metals. This work combines sequences of different promoters, metal-binding proteins (Fe^{2+}, Pb^{2+}, Na^{+}, and Cl^{-}), ribosomal binding sites, fluorescent reporter protein, terminators, and a synthetic riboswitch, in synchronization with a protein (TonB) playing the role of an iron-ion channel.

The objectives of this study were to develop a biosensor engineered and assembled for potential sensitive detection of bivalent cations such as iron and lead, and to use a ribosomal switch to regulate the specific detection of these metals. The detection of these cation-binding proteins (Fe and Pb) would signal the properties of *E. coli* colonies that have been modified to respond to them and would increase the fluorescence of the protein reporter.

2. Materials and methods

2.1. Bacterial culture and competent cells

Bacterial strains of *E. coli* DH10 were obtained from Invitrogen (USA), grown in Luria-Bertani (LB) medium, and incubated at 37 °C. These bacterial strains were used as competent cells in which different genetic parts were assembled.

2.2. Genetic parts

The DNA biosensor device was assembled in *E. coli* competent cells to construct the molecular bacterial metal sensor. The rpoS ribosomal switch and TonB transporter ion channel protein sequences were obtained from Gene Bank: D26134.1 and ACB93044.1, respectively. The riboswitch rpoS gene was synthesized with the addition of Hind III & Kpn I sites at 5′ and Hind III & Xho I sites at 3′. The synthetic gene was sub-cloned into EcoRV site of pBSK II vector. Other synthesized gene parts were also assembled into the device, including Fe promoters, metal-binding proteins (Fe, Pb), yellow fluorescent reporter protein (YFRP), and a ribosomal binding site (RBS) J61100 taken from the iGEM registry parts (MIT, MA). The sequences of these gene parts were also obtained from the Gene Bank in the Pubmed database and synthesized by CloneTEX company (Austin, TX).

Different genetic parts and/or sequences were tested and purified through DNA extraction and electrophoresis. DNA extraction

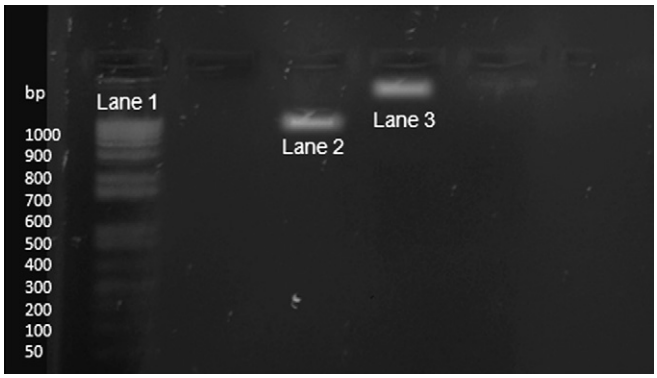

Fig. 2. Gel of DNA of the two biosensor devices grown without iron, after electrophoresis. The photo shows, from left to right, lane 1, DNA marker (1000 bp); lane 2, DNA of biosensor device without ribosomal switch showing lower molecular weight than the biosensor device with ribosomal switch; lane 3, DNA of biosensor device with ribosomal switch showing higher molecular weight than biosensor without ribosomal switch.

was performed from single bacterial colonies using WIZARD Plus Minipreps DNA Extraction and Purification System (Promega), followed by electrophoresis.

2.3. Enzyme digestion, assemblage of biosensor, and ligation

Digestion reactions were carried out at 37 °C using New England Biolab restriction endonuclease enzymes and buffers, and each single gene was digested with compatible restriction sites from the multiple cloning region of the pBSKII plasmid. Using 4 μl of 10× buffer, 4 μl of the template DNA, 0.4 μl BSA, 2 μl of each restriction enzyme, and 27.6 μl ddH_2O, a final volume of 40 μl was incubated on a heat block at 37 °C for 2 h to ensure complete digestion. Alkaline phosphatase treatment was added one hour into incubation. Samples were then run in an electrophoresis gel to separate the DNA fragments using a molecular ladder, allowing identification of the gene of interest. Gel bands were isolated and purified using the NEB gel purification kit.

The assemblage of genes was carried out according to Biobrick protocol into a pBSKII high-copy plasmid, and selected genes were ligated into the multiple cloning region of the pBSKII plasmid. After the different parts were tested and/or purified, they were assembled in a proper sequential order in competent *E. coli* cells, thus constructing the DNA biosensor device. The sequential order was as follows: T3 promoter + Fe-promoter + Ribosomal switch rpoS + Ion channel TonB + Fe-binding protein + Pb-binding protein + yellow fluorescence protein reporter + terminator (Fig. 1).

Ligations were performed following the Quick Ligation Protocol of New England BioLabs using T4 ligase. Fifty ng of backbone vector were combined with a 3-fold molar excess of gene insert. Volume was adjusted to 10 μl using ddH_2O, after which 10 μl of 2× Quick Ligation Buffer was added, along with 1 μl of T4 DNA Quick Ligase; the combination was mixed thoroughly. Each reaction was centrifuged briefly and incubated at room temperature (25 °C) for 5 min before transformation of competent cells or storage at −20 °C.

Two types of DNA biosensor devices were constructed, either with or without the ribosomal switch sequence. Each type of device was compared to the control, which consisted of non-transformed competent *E. coli* DH10 cells.

2.4. RNA extraction

Bacterial cell cultures were harvested at 24 h and subjected to RNA extraction, following QIAGEN protocol (CA, USA). Bacterial cells were harvested by centrifuging 2 mL of cell culture at 5000 × *g* for 5 min at 4 °C and decanting the supernatant. Then 350 μl of Buffer RLT was added and resuspended by vortexing for 10 s. After transferring 700 μl of lysate to an RNeasy spin column, the material was placed into a 2 mL tube, the lid was closed gently, and the mixture was centrifuged at 8000 × *g* for 15 s. The flow-through was poured off, and 700 μl of Buffer RW1 was added to the spin column; again, the lid was closed gently and the mixture was centrifuged at 8000 × *g* for 15 s. The flow-through was poured off again and 500 μl of Buffer RPE was added to the spin column, the lid closed gently, and centrifuged at 8000 × *g* for 15 s. The flow-through was poured off a third time and the above washing step was repeated. The spin column was placed into a new 2 mL tube, 40 μl of RNase free water was added to the spin column membrane, and the mixture was centrifuged at 13,000 × *g* for 1 min. The RNA concentration of the device or transformed cells of the biosensor were quantified using triplicate 1-mL samples and measured in a nano-spectrophotometer (nanoDrops-UV/vis spectrophotometer-MET-Limited), for each iteration.

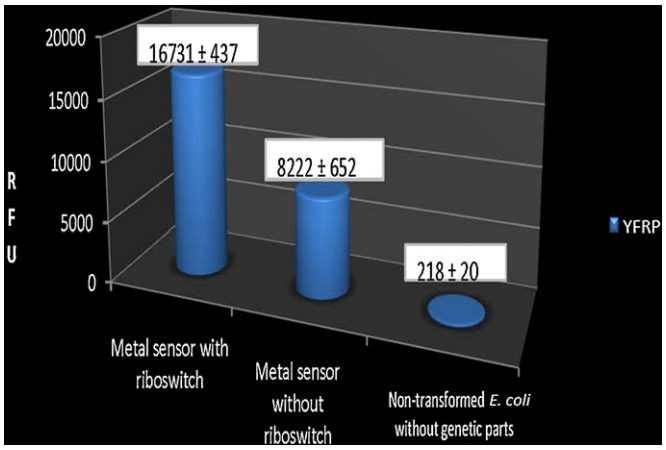

Fig. 3. The detection ability of developed biosensor, grown without iron, showing measured relative fluorescent units (RFU) among the biosensor for metals with riboswitch translational regulation of YFRP, biosensor without ribosomal control, and control (non-transformed) *E. coli* cultures.

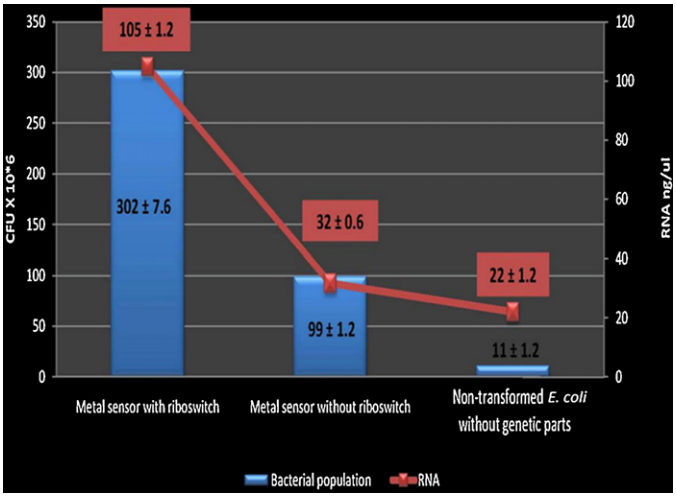

Fig. 4. The detection ability of developed biosensor, grown without iron, showing colony-forming units and RNA concentrations of the biosensor for metals with riboswitch translational regulation of the constructed device, biosensor without ribosomal control, and control (non-transformed) *E. coli* cultures.

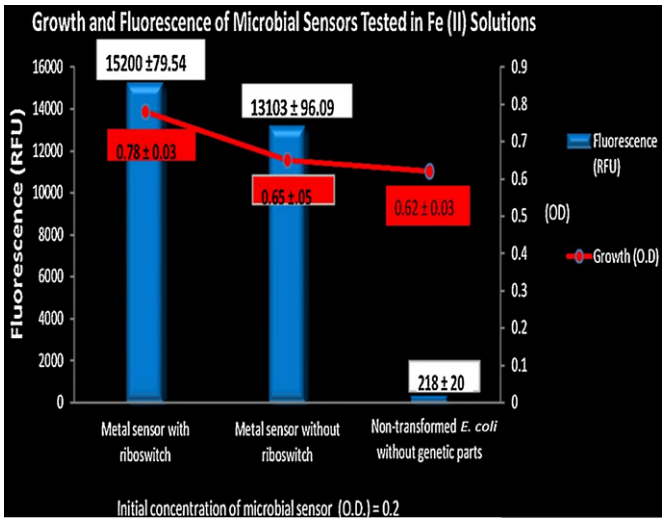

Fig. 5. The detection ability of developed biosensor, grown in iron (5 μg/ml), showing growth and fluorescence (RFU) of the biosensor for metals with riboswitch translational regulation of the constructed device, biosensor without ribosomal control, and control (non-transformed) *E. coli* cultures.

2.5. Fluorescence measurement

Fluorescence of the bacterial sensor was determined after 24 h of growth using a 20/20 luminometer (Turner Biosystems, USA). Triplicate 100-μl samples of bacterial sensor were used for determining fluorescence for each iteration, and the results were expressed in relative units of fluorescence (RFU).

2.6. Test of bacterial sensor in metal solution

Detection efficacy of the bacterial sensor was measured in iron or lead sulfate solutions at 5 μg/ml concentration. These tests were done using bacterial sensors at concentrations of 0.2 optical density (O.D.), measured by standard spectrophotometry method, UV/vis spectrophotometer (BECKMAN, USA).

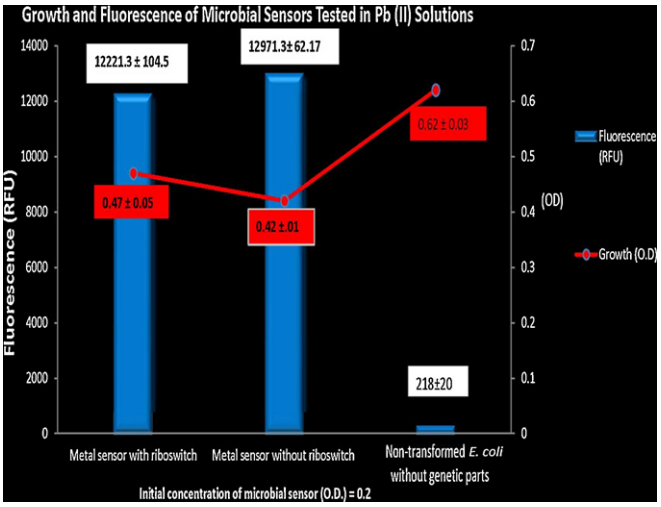

Fig. 6. The detection ability of developed biosensor, grown in lead (5 μg/ml), showing growth and fluorescence (RFU) of the biosensor for metals with ribosomal switch translational regulation of the constructed device, biosensor without ribosomal control, and control (non-transformed) *E. coli* cultures.

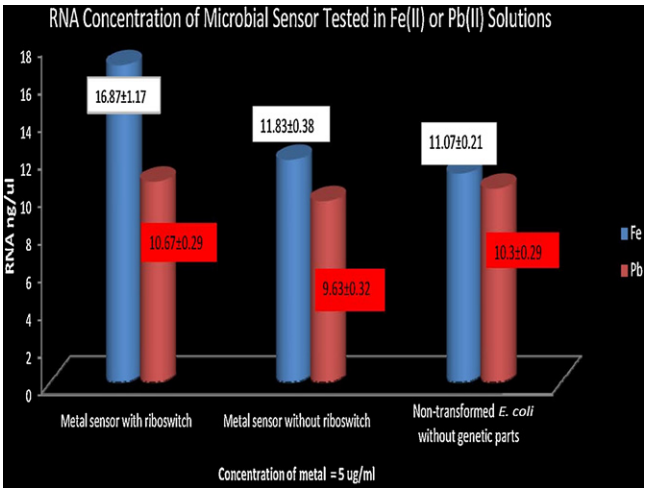

Fig. 7. The detection ability of developed biosensor, grown in iron or lead (5 μg/ml), showing RNA concentrations of the biosensor for metals with riboswitch translational regulation of the constructed device, biosensor without ribosomal control, and control (non-transformed) *E. coli* cultures.

3. Results

Effective assemblage of the microbial biosensor was confirmed by the tested ribosomal switch–ion channel synchronization in relation to the iron and lead protein interaction and the level of fluorescence of the yellow reporter protein. Fig. 2 shows the single DNA band of the ligation of parts of the DNA metal biosensor device with and without a ribosomal switch after electrophoresis, corroborating the effective assemblage of the parts of the microbial biosensor device (gel for the Pb ion channel is not shown). This result shows the efficacy of the constructed bacterial biosensor with ion channel containing a ribosomal switch compared to the bacterial sensor with ion channel that did not contain a ribosomal switch. The effective assemblage of the parts of the microbial biosensor device is demonstrated in cases of both Fe and Pb ions; the presence of the target ion, specifically Fe, was efficiently detected by the DNA metal biosensor containing the ribosomal switch through analysis of measured parameters. This may indicate the effective interaction between the ribosomal switch and ion channel.

This detection was reflected by the increased growth (measured in CFU, O.D.) of the bacterial sensor (Figs. 4–6 and 8). Growth was higher for the bacterial sensor with the ribosomal switch–ion channel genes that were grown in an iron solution. These agar plates were fully covered by bacterial colonies (Figs. 5 and 8c), whereas this was not the case for the bacterial sensors without the ribosomal switch–ion channel genes (Fig. 8d), grown either with or without the lead solution (Fig. 6). Concentrations of RNA were also higher in the bacterial sensor containing the ribosomal switch–ion channel genes compared to the bacterial sensor without the ribosomal switch, regardless of whether they were grown in the iron solution, the lead solution, or with neither. Additionally, the RNA concentration was higher in the bacterial sensor grown in iron or lead solution compared to the bacterial sensor grown without iron or lead solution (Figs. 4 and 7). Perhaps the higher RNA concentration in the bacterial sensor with the ribosomal switch confirms that the ribosomal switch containing an aptamer, which responds to the iron ions acting as a ligand, synchronizes with the iron ion channel, TonB (Cuero et al., 2003; Cuero and Ouellett, 2005; Quintero et al., 2007) to enhance the specificity of the molecular biosensor. The slight decrease of RNA concentration in the bacterial sensor could be due to the effect of the iron solution in the bacterial cell. The iron may have caused an ionic cellular shock during the first short period of bacterial cell growth. Perhaps RNA concentration would increase

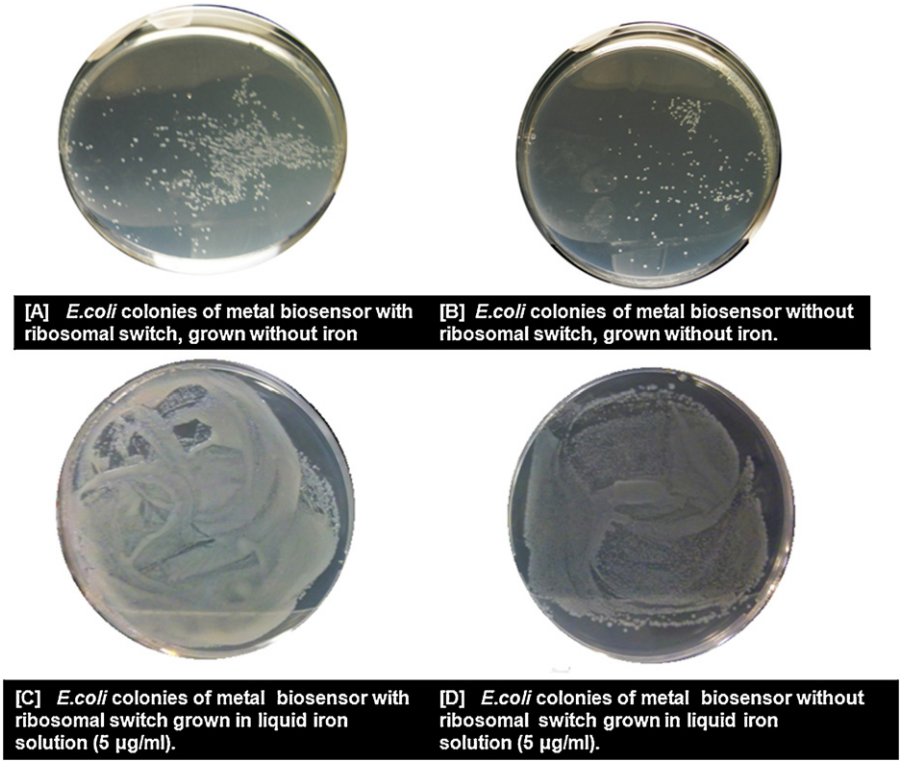

Fig. 8. *E. coli* colonies of metal biosensor with [A] or without [B] ribosomal switch, grown without iron. *E. coli* colonies of metal biosensor with [C] or without [D] ribosomal switch, grown in iron (5 μg/ml). Bacterial sensor colonies cover the surface of the agar plate, but bacterial sensor with ribosomal switch–ion channel grown in iron showed noticeably more growth.

again, after a longer period of bacterial growth, when the cell populations have adapted completely to the metal milieu (Cuero et al., 2003).

The bacterial sensor with ribosomal switch–ion channel, grown either with or without iron solution, also exhibited brighter fluorescence compared to the bacterial sensor without ribosomal switch, grown with or without lead solution (Figs. 3, 5, 6, 8a and c). This result corroborates the effect of the ribosomal switch in enhancing the detection of metal in the bacterial DNA metal sensor.

Fig. 4 exhibits a clear, direct correlation between concentration of RNA and bacterial population (CFU) of the device and the type of biosensor. The DNA metal biosensor with ribosomal switch showed higher CFU, corresponding to a higher RNA concentration, while the DNA biosensor without ribosomal switch showed both lower CFU and RNA concentration. These results also correlate to the fluorescence of the YFRP, expressed in relative fluorescence units (RFU); the DNA biosensor device containing the ribosomal switch showed higher RFU (16,731), followed by the DNA sensor without ribosomal switch (8227 RFU), and then the control, which showed the lowest fluorescence (218 RFU) (Fig. 3).

4. Discussion

The assemblage of the standardized biobrick parts with the synthesized sequences, such as metal-binding proteins, TonB ion channel, and rpoS ribosomal switch, was achieved effectively as reflected in their sensitivity to the metal ions. This effective assemblage of the biosensor parts is due to the appropriate design of the position and/or sequential organization of the parts within the device or biobrick (Quintero et al., 2007). The fact that the ligation showed a higher position of the corresponding DNA band indicates that all parts were assembled. Fig. 2 shows the electrophoresis gel corresponding to the metal biosensor device containing all the parts along with rpoS, thus demonstrating that all the parts of the device were ligated. (Other biosensor devices were not subjected to gel electrophoresis).

Genes are made of hundreds of atoms (Kauffman, 2000), therefore, it is rational to think that the interactions of the parts could be influenced by electrochemical principles of the atoms (Weast et al., 1989; Kauffman, 2000) at the binding sites.

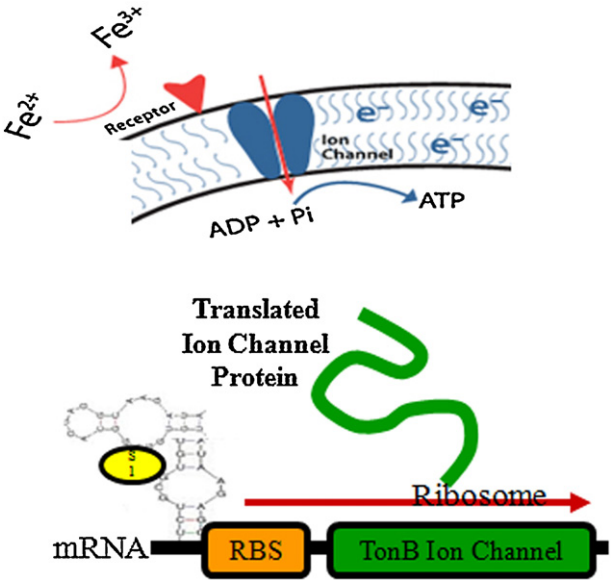

Fig. 9. Graphic illustration of the ribosomal switch–ion channel synchronization. When bound to the S1 regulatory protein, the rpoS riboswitch undergoes a change in tertiary structure, allowing the ribosome to bind to the RBS and initiate translation of the downstream TonB ion channel. The translated voltage-gated ion channel becomes embedded in the cellular membrane, allowing bivalent cations to pass into the cell, becoming oxidized as they enter, aiding in the synthesis of ATP.

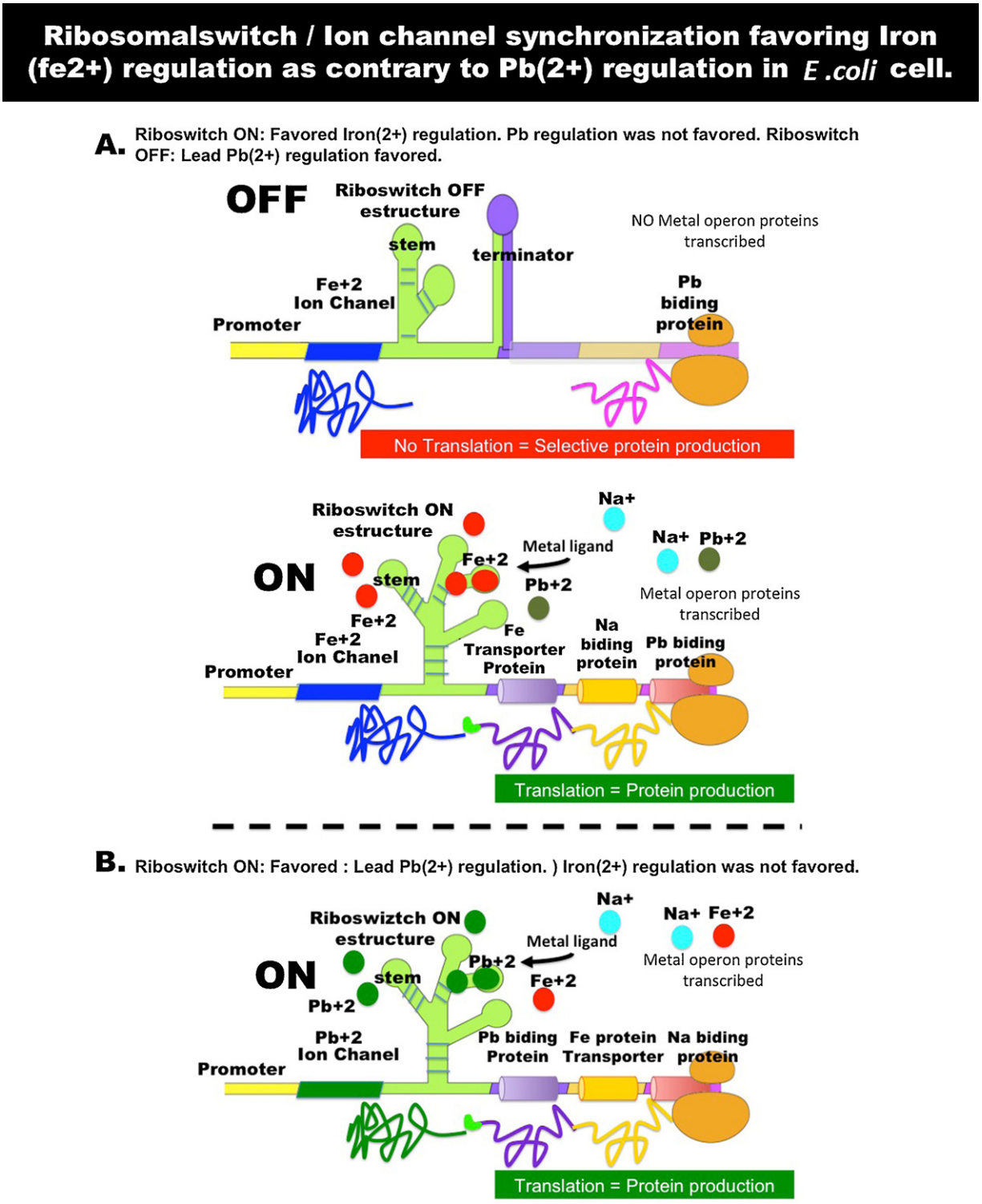

Fig. 10. Graphic illustration detailing the mechanics of the ribosomal switch–ion channel synchronization. (A) The change in rpoS tertiary structure when bound to ligand metabolite favoring iron (Fe^{2+}) regulation. (B) Ribosomal switch favoring lead (Pb^{2+}) regulation.

The correlation of selected different parameters, CFU, RNA, and intensity of the fluorescence (RFU) of the YFRP, measured the efficacy of the assembled biosensors in relation to the presence of the ribosomal switch. Fig. 4 demonstrates the correlation between the concentration of bacterial cells with the biosensor and the concentration of bacterial RNA; this shows a higher CFU (3×10^8 cells/ml) corresponding to the higher RNA concentration (105 ng/μl) in tested biosensors containing the ribosomal switch, and the lowest CFU (1×10^7 cells/ml) corresponding to the lowest RNA concentration (22 ng/μl) in tested biosensors without the ribosomal switch. These results are confirmed by the brighter yellow fluorescence of the colonies seen in the biosensor containing the ribosomal switch, compared to the less bright fluorescence of the bacterial device without the ribosomal switch (Fig. 8) and no fluorescence of the colonies from the control without the assembled parts. Likewise, these fluorescence data correspond to fluorescence units (RFU) of

the YFRP, when determined photo-electrically in the 20/20 luminometer. Thus, higher fluorescence (16,731 RFU) was exhibited by tested metal biosensors with the ribosomal switch, compared to metal biosensors without the ribosomal switch (8222 RFU) and to control or bacterial cells without assembled parts (8222 and 218 RFU, respectively).

Metal ions have shown a direct effect on enhancing gene expression, and RNA concentration in microbial cells (Cuero et al., 2003; Cuero and Ouellett, 2005). However, the interaction of these metal proteins and their mode of regulation in relation to the detection of different types of metals were not previously elucidated. This synthetic biology approach has demonstrated the specific interactive effects of the ribosomal switch and ion channel proteins on regulating metal-binding protein expression by the biosensor (Figs. 3–8). These effects provide selective detection of the metal ions used in our system.

Results of this investigation clearly show the effect of the ribosomal switch in enhancing fluorescence of the biosensor reporter protein. It is likely that the ribosomal switch is mediating ion channel protein activity and/or regulating expression of the metal-binding proteins at the translation level. The ribosomal switch rpoS, which responds to oxidative stress such as the presence of metal ions, changes its tertiary structure when bound to the S1 sigma factor due to the methylation of its aptamer, and increases its affinity for ribosomes to bind with the ribosomal binding site immediately downstream (Hengge-Aronis, 2002). This translation regulation of the production and function of specific proteins seems to be mediated through synchronization between the ion channel TonB and ribosomal switch rpoS (Figs. 9 and 10).

Our results show higher fluorescence of the YFRP, as well as a higher number of fluorescent biosensor colonies containing the ribosomal switch, compared to biosensors without the ribosomal switch (Figs. 3–6 and 8). This suggests that the ribosomal switch in synchronization with the ion channel regulates expression and/or signaling of the metal-binding proteins.

Additionally, these signals may reflect an oxidative stress, which may have turned on the translation domain of the ribosome. However, this could depend on the availability and/or types of metal-binding proteins (Figs. 9 and 10). Bacterial RNA binding proteins with sensing properties in the regulatory system have been recognized. Ribosomal protein operons are repressed by individual ribosomal proteins when the amount of free proteins exceeds the amount of free ribosomal proteins. Consequently, RNA will exhibit signals that more production of ribosomal proteins is not needed (Grundy and Henkin, 2006). This may also explain how the synchronization of this ribosomal switch with an ion channel such as TonB had an effect on the mechanism of the metal-binding protein expression in our experiments.

Some of the regulatory elements of the RNA structure that affect the initiation region of translation or the target gene under normal growth conditions may unfold to permit a downstream translational sensing response (Grundy and Henkin, 2006). This seems to be the case in the present investigation with metal-binding proteins, in which the bacterial sensor with the ribosomal switch and ion channel protein showed more colony populations and higher protein fluorescence (Figs. 3–6 and 8).

This observation also confirms the successful assemblage of the genetic parts of the biosensor, as well as the expected increased metabolism of the bacterial cells containing the ribosomal switch, in which there was a corresponding higher production of RNA and translated proteins. This result was corroborated by both higher numbers of bacterial biosensor colonies and higher fluorescence of the YFRP (Figs. 3–6 and 8). Certain amino acids, histidine and aspartate, are phosphorylated to form a two-component system. The first system is an input-sensing histidine-kinase that responds to specific stimulus. Then the kinase transfers the acquired phosphate to a residue aspartate, thus creating a second component. Sometimes the two components become integrated into a single system and are able to detect more complex signaling. In the same way, the biosensor enables bacterial cells to detect various environmental inputs, such as pH, nutrients, salinity, and temperature (Mijakovic, 2010).

We are carrying out further experiments to test the sensitivity of the different biosensors constructed, using varying concentrations of different types of metal ions. Also, new biosensors containing different ion channels and ribosomal switches are being assembled. One of the objectives of this new investigation is to synchronize ion channels with ribosomal switches to achieve better regulatory control of metals, and even to achieve specific selection of metals. We will explore different types of metal-binding proteins, based on the active binding site, and we are developing a computational model to predict the detection and concentration efficiency of synthetic biology biosensors for different metals.

Acknowledgments

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