Review article

Analysis of selected toxic metals using bacterial biosensors and their macromolecular derivatives

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Abstract: *This short review presents a number of bacterial transcription mechanisms dependant on metal occurrence in growth environment. Metallic ions such as mercury, cadmium or zinc evoke genetic response, which then can be engineered and utilized to reprogram cells. Recombinant biosensors could be applied in detection and quantification of trace amounts of toxic metals. The article presents a few examples of such use and also touches upon other related approaches where sensitivity to metals was a backbone for an idea to measure metal concentrations.*

Key words: *biosensors, gene regulation, metals, detection, GFP.*

Introduction

All cellular functions are tightly controlled on molecular level. In order to preserve cell viability, the ongoing transcription and translation processes must remain dynamically responsive to variable extracellular stimuli. The incoming signal detected on the external surface is reflected in intracellular occurrence of numerous inducible regulators. Silencing and enhancing sequences present within genome serve as binding *loci* to repressors and activators, agents either inhibiting or accelerating specific gene expression. For the most part, gene transcription is covered by RNA Polymerase (RNAP), where the enzyme is complexed with σ70 factor. While σ70 navigates to a subset of genes crucial for regular cell activities, another sigma factor, known as σ54, is ascribed to some of these associated with defence against chemical stress [1-2]. In this short review an attempt is made to elucidate a few detox mechanisms where σ70 or σ54-RNAP complex participation is implicated in metal transport in and out of cell. Next, a number of examples is presented where this particular or analogous knowledge of reactivity to metals was put in practice in an approach to create robust biosensors.

Metals migration into the natural environment is accelerated by industrial and agricultural activities. The most detrimental elements, purely xenobiotic for a living organism, include mercury, led, cadmium. Other metallic toxicants encompass arsenic, chromium, cobalt, copper, nickel, molybdenum and zinc, most of which are harmful in excessive doses [3-5]. Their influx into the bacterial cell must be tackled before it suffers necrosis and it is performed in the course of

a number of intrinsic mechanisms. These proceed through transporting adverse agents out of the cell.

Transcriptonal response

Grouped as a *MerR family* due to the similarity shared in N-terminal DNA binding region, a number of proteins are involved in mercury resistance acquisition. A subgroup of thereof presents affinity in C-terminal inducer binding sequence and is responsive to metals. Mercury resistance genes are clustered in an operon referred to as *mer*, found in species-specific transposable elements. Downstream of the promoter/operator region, the operon contains various sets of structural genes, hallmark of a given species. Shigella flexteri's enlists MerT (transporter), MerP (periplasmic protein), MerC/F (additional transporter), MerA (mercuric reductase), MerD (putative reductase) and MerE (putative futher transporter). In general, though, we distinguish functional groups such as regulatory, transporting and reducing genes. MerR-family member regulatory protein named MerR oversees expression of the structural genes in the *mer* operon. Depending on the C-terminus variant, it exhibits sensitivity to organomercurials or inorganic mercury compounds in which case additional organomercurial lyase is needed in amongst detoxifying genes. When bound to DNA at the operator region, operon transcription is inhibited. This is reversed when mercury binds MerR, releasing transcription hindrance for σ70-RNAP complex. With cell now being able to conduct detoxification, Hg^{2+} ions are bound to periplasmic MerP and transferred onto the consecutive transporting proteins (i.a. MerT) until they reach mercuric reductase and exit cell as volatile Hg^0. It is hypothesized that once the cell is clear of mercury, Hg^{2+}-$MerR_2$ complex is superseded with an unbound form of MerR or a repressive substitute, MerD. MerR family regulators are well conserved throughout Gram-negative bacterial genera and so is the mechanism of action exhibited by its member proteins [6-7].

Nickel is another element whose migration in and out of cell is tightly controlled. NikR is a transcription repressor for *nik* operon, with a role analogous to this of MerR. Contrary to mercury, though, nickel uptake is a desired process, but only to a certain extent. Otherwise it becomes harmful. Nik operon codes for 5 proteins – NikABCDE. NikA is a soluble periplasmic protein that binds nickel. NikB and NikC are hydrophobic transmembrane proteins. NikD and NikE are involved in coupling energy from nucleotide hydrolysis to the transport process through the membrane. The five co-operate to satisfy cell's demand for physiologically essential nickel. Downstream of the last component of this structural set, a regulatory protein is transcribed. When intracellular nickel concentration becomes too high, inactive NikR binds nickel ion and anchors on operator/promoter sequence of nik operon to shut off further uptake. Apart from its induced occurrence through expression from the promoter of nik operon, this repressor also undergoes constitutive basal level of transcription, independently of nickel level, to ensure rapid response should excessive Ni^{2+} appear. While

there is a number of nickel-dependant enzymes in plentiful microorganisms, Escherichia coli is the only one where this highly specific nickel transport system was described [8-10].

The aforementioned negatively regulated mechanisms are achieved with a use of σ70 subunit. However, some of the other metal-induced pathways are strictly σ54-dependent. This is the case for *Escherichia coli* two-component regulatory system ZraS/R, formerly known as HydH/G, whose role is to regulate tolerance to zinc. Poorly explored as it is, ZraS appears to act as a putative metal-responsive sensor kinase, located at the membrane. When exposed to zinc, it passes the molecular signal onto its cognate response regulator, the transcription activator ZraR, which, once phosphorylated, binds enhancer site preceding a gene sequence that codes for ZraP protein. ZraP is a periplasmic zinc-binding protein whose expression is undertaken by σ54-RNAP complex. Its functional significance is elusive. A role of preserving metal homeostasis used to be ascribed to ZraP, but so far not evident. It does, however, act as a zinc-dependent chaperone suppressing thermal aggregation of proteins in cellular stress conditions [11-13].

Two-component systems are a common solution when it comes to metal sensing. It is also the case for genes upregulated in response to ionic copper, where several regulons can be distinguished that account for to date poorly explored, holistic systemic defence mechanism. CusCFBA is an example of an operon implicated in homeostasis and copper circulation processes. Its transcription is launched as a consequence of the action from two upstream regulators, a response factor (CusS) and its kinase activity substrate (CusR). The latter binds in vicinity of cusC gene sequence to activate transcription of genes clustered in a corresponding operon and ultimately form a tripartite transporter. It is one of the two chromosomal copper tolerance systems identified in *E.coli,* alongside the one where both copper-inducible CueO periplasmic oxidase and CopA transporter are expressed and stimulated by CueR regulator [14-16].

Biosensors responsive through genetic regulatory networks

Sensitivity to the presence of metallic ions is a bacterial advantage that could be used for better detection, to ensure safety and eliminate poisoning hazards in possible carrier matrices. Having identified responsive molecular dependencies in bacterial genotype, like these described above, an advance in genetic engineering can be further directed to combine desired transduction switches with reporting molecules as a means for bio-quantitative analysis. Traditional detection techniques (AAS, ICP-MS) rely on machines; they are complex as far as their use and sample preparation go, which makes them inadequate for use outside of well-equipped scientific laboratories. This is where prospects open for biosensors [17].

E. coli BL21 was chemically transformed, *sensitized* to cadmium to produce fluorescent output signal. This was done by incorporating into a pET30b vector both ectopic Cd^{2+}-responsive cadR gene from *P. aeruginosa* and a sequence

coding for reporter GFP gene, extracted from pEGFP-1. This simple cloning experiment allowed to witness UV-induced fluorescence indicated at the presence of cadmium, but it was not further optimized for quantitative analysis [18]. More elaborate evidence for this idea of biosensor comes from research, where transformation served the purpose of detecting inorganic forms of arsenic As^{+3} and As^{+5} as well as antimony Sb^{+3}. ArsR is an arsenic-dependent regulatory repressor, a part of *ars* operon, holding the cell back from the formation of efflux pump transporting this element. Authors coupled the promoter site from *ars* operon derived from S. aureus-isolated pI258 vector with GFP in a pVLAS1 plasmid. Repression abrogated by the presence of metallic inducers inside the cell allowed for fluorescent emission and its subsequent quantification, in a manner proportional to the extent of induction. Study was conducted with the use of DH5α *E.coli* strain analyzed on spectrophotometer and fluorometer simultaneously against blank-vector transformed cells. With a high level of specificity towards only the three elements, 0.4 and 0.1 μM As^{+3} was the minimal concentration detected in a 2- and 8h induction period. For As^{+5} these values equal 1 and 0.1 μM, respectively and for Sb^{3+} it is 0.75 and 0.1 μM. The biosensor viability was additionally confirmed in groundwater analysis [19]. Another research utilized mercury resistance operon regulatory sequence. *E.coli* JM109 were transfected with pmerGFP, a pGFPemd-b vector with inserts of MerR repressor as well as *mer* promoter/operator sequences and GFP. Then, they were immobilized into two coating latex layers. The best output signal was obtained from cells located in between two layers of coating. The minimal detectable mercury chloride concentration tested in this assay was 2μM and fluorescence signal depended on temperature conditions [20].

Use of living biosensors is confined by parameters that otherwise would not matter. Extent of toxicity determines the range of tolerable concentrations i.e. interactions with mercury occur at a threshold thousand times lower than most other elements. Minimal growth inhibitory concentration for *E.coli* is 11.5 μM $HgCl_2$ [21]. Often, metals come in an unacquirable, complexed or adsorbed form. Bioavailability, proportion of total metals that are available for incorporation into biota [22], is what must be taken in consideration when designing an assay. Complex organic matrices, even if positive for metal contaminants, may have them *trapped* so as an ion is no longer accessible and affects biosensor in no way. Bioavailability is influenced by chemical composition of environment and its dynamic physicochemical properties [23]. Dissolved free ionic metals used in initial laboratory assays are usually well, often the most, bioavailable [24]. Natural phenomena of particulating and complexing can shift this property in any direction with different intensity. For example mercury complex with cysteine displays enhanced Hg^{2+} bioavailability (toxicity augmented) [25], while others like chelators such as dimercaprol and succimer, tune it down [26].

Other examples of biosensing

While the foregoing efforts are aimed to accomplish a feedback between metal presence and transcription of a labeled activator, an alternative proteomic approach can be taken where an analytical signal derives from an element binding to a peptide. A cadmium binding peptide fused to GFP was used in quantitative analysis. Enhancement of cells' fluorescence reported was proportional to the concentration of cadmium cells were exposed to in a linear manner, starting as low as 0.5 μM. This way, cadmium ions activated fluorescence up to 0.5 mM Cd^{2+} in a 180 min run. Reading was evaluated with wild-type GFP expressing cells as a reference and proved *E. coli* to be a successful messenger for trace cadmium contamination [27]. For signaling efficiency analysis, it would be interesting to see the results plotted against transcriptomic approach assay, where promoters of genes upregulated directly as a response to occurrence of cadmium would be coupled to GFP. While cadmium presence incurs expression changes to 674 transcripts, regulators of metal-transporting proteins involved in efflux systems would suit this type of examination, assuming their specificity to cadmium or absence of co-transported metals in induction medium [28].

In some cases there is even no necessity to search for an appropriate binding agent to be coupled with a generic reporter. The GFP itself can play both these roles, as it turns out from mutagenesis study, where introduction of a cysteine residue at position 205 turned this protein into a biosensor for mercury. Thus, a binding site is created next to chromophore with sulfhydryl groups available for mercury to access. When mercurated, altered GFP exhibits shift in its absorbance and fluorenscence spectra. Concentration as low as 2 nM $HgCl_2$ was reflected in fluorescence quenching of the modified protein [29].

Alternatively, toxic metal presence could be measured indirectly through examining toxicity from the matrix cells suffer exposure to. Metabolic activity itself becomes a reporter here. It reflects growth medium properties and is closely correlated with luminescent properties of lux-transformed cells. The more toxic environment is, the more substantial quenching of light emission is observed. This is rather qualitative analysis and assumes no detrimental factors other than metal ions affecting culture growth in the medium [30]. In another research, authors used luminescence dependant on zntA and copA promoters, whose transcription is initiated as a cellular response to zinc/cadmium/lead/mercury and copper/silver, respectively. ZntR and CueR were their immediate transcription activators. This time, positively regulated response increased emission of light as higher metal concentrations were used as inducers. The lowest detectable response from zntA promoter was reported for cadmium at the concentration of 10 nM, whereas copA was expressed at the minimum of 0.1 μM silver [31].

Conclusion

While the potential in successful use cannot be questioned, instrumental methods of chemical analysis may well be supplemented with biosensoric units genetically improved to transduce analytical signal in a measurable manner via reporter genes such as GFP, lacZ, lux or luc [17, 32]. The pathways and methods described in this paper exemplify how bacteria are equipped with natural sensing tools. However, it is necessary to point out bacterial analysis only relates to bioavailable forms of metals, rather than their total amount. Having determined metals as a hazard to our health that often occurs in trace amounts as well as being able to distinguish molecular elements participating in consecutive stages of detection *in vivo*, we are competent to apply this knowledge for a tangible benefit that goes beyond academic science. Each type of such biosensors is characterized by its efficiency span, with upper limit determined by cell viability and lower limit being reflection of sensing capabilities. Most experiments quoted above are based on a roughly similar pattern. However, each must be evaluated separately and stands for an individual approach that is difficult to draw comparisons with. This is due to numerous variables that were applied and are ultimately reflected in final outcome. Bacterial species, or even their strain, genes used for transformation, vector carriers, intensity of induction and culture conditions all make up a certain context for reproducibility and feasibility. Limit of detection, analytic signal resolution and measurable span of metal concentrations are the universal criteria for reference and they must not be considered in isolation from overall design of a particular report.

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