Toward understanding metal stress in environmental microbial flora

J. Guzzo, C. Diorio, D.C. Alexander, M..S. DuBow

Department of Microbiology & Immunology, McGill University,3775 University Street Montreal, Quebec, Canada H3A 2B4

ABSTRACT

All organisms have evolved the ability to measure changes in their environment and, in most cases, strategies to deal with these changes. The triggered molecular events often involve transcriptional activation or repression of a set of genes whose products allow the reorientation of cell physiology to cope with the environmental change. We have used luciferase gene-fusions in order to discover these genetically-programmed responses in *Escherichia coli* in a more systematic fashion. We have searched for genes whose expression is altered upon cellular exposure to environmentally-relevant concentrations of priority environmental pollutants such as arsenic, aluminum and selenium. We have identified genes whose expression is induced by these (and other) chemicals and found that many have no known homologues in the databases from which their function or regulation can be discovered. These clones can be used as exquisite biosensors to measure (via the induction of luminescence) the bioavailable concentrations of these chemicals in the environment, as well as determine their affects on cell physiology and the cell's response to them at the molecular level. Approaches such as these will also allow the characterization of how bacteria respond to these environmental pollutants and ultimately, how the micro-flora can be affected and protected from these environmental stresses.

Introduction

Many ecosystems are currently being threatened by the industrial release of metal-, metalloid-, and organometal-containing compounds. Furthermore, these compounds most likely alter the diversity and physiology of communities of microbial flora in these contaminated environments (Fig.1). To understand the molecular and physiological basis of these responses, we have developed a system to monitor transcriptional changes of individual genes which may be expressed to help microorganisms cope with the chemically-induced stress [1, 9]. We chose to study these responses in *Escherichia coli*, the common enteric bacterium, due to its superb *in vivo* and *in vitro* genetic manipulations and the wealth of information concerning its genetics and physiology. Measurement of these transcriptional alterations in quantifiable units was made possible by inserting the promoterless *luxAB* transcriptional "reporter genes" from the marine bacterium, *Vibrio harveyi*, into individual genes. The *luxAB* genes encode luciferase, a mixed function oxidase which requires an aldehyde and a diflavin mononucleotide in the presence of oxygen, and results in a carboxylic acid, a flavin mononucleotide, water, and light at 490nm [12]. When

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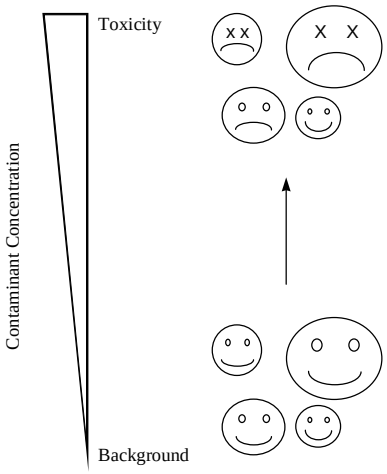

Fig. 1. Diagram representing the diversity and relationships between communities of microbial flora in the presence of increasing concentrations of an environmental contaminant. Many contaminants, such as trace elements like selenium, are known to be essential at background concentrations for prokaryotes and eukaryotes [14], depicted here as happy faces. However, at higher concentrations, many cellular disorders can appear, including cell death [14], depicted by sad faces.

a gene, containing the *luxAB* reporter genes, is differentially expressed in response to the chemically-induced alteration in cell structure and/or function, the change in expression of this gene is easily assayed by coexpression of *luxAB*, with expression proportional to light emission from the clone containing the transcriptional gene fusion [9].

To identify genes involved in these stress responses, the *luxAB* genes were cloned into a modified Tn*5*-*tet* transposon, which was used to randomly insert the reporter genes in single-copy into the *E. coli* chromosome (Fig. 2A) [9]. In doing so, approximately 3000 single-copy luciferase gene fusion were created. These clones were then screened in the absence and presence of increasing concentrations of various metal-, metalloid-, and organometal-containing compounds (Fig. 2B). Clones that exhibited altered gene expression in the presence of these compounds were detected by replica-plating onto media containing increasing concentrations of compound, adding aldehyde to the covers of the petri dishes, exposing the dishes to X-ray film, and looking for concentration-dependent changes in luminescence. Using this screening procedure, numerous clones were identified and genes responding to aluminum, arsenic/antimony, selenium, and organotin compounds are currently being characterized (Fig. 2C).

Another important application for these *luxAB* gene fusion clones is the rapid and cost-effective monitoring of environmentally relevant concentrations of these substances in soil and water ecosystems. Many biologically-based sensors have been previously described. For example, the first enzyme-based sensor for glucose was described in 1963 [5]. More recently, genetically-engineered microorganisms have been employed to determine biologically relevant concentrations of mercury and naphthalene [11, 13]. Using the luminescent bacterial biosensors created in our laboratory, we can rapidly quantify bioavailable concentrations of aluminum, arsenic, selenium, and organotin compounds in soil and water ecosystems (Fig. 2C) [2, 6]. Bioavailability is extremely important since compounds may exist in many forms in these ecosystems and not all of these forms will be biologically active. Furthermore, only a portion of the net concentration of these

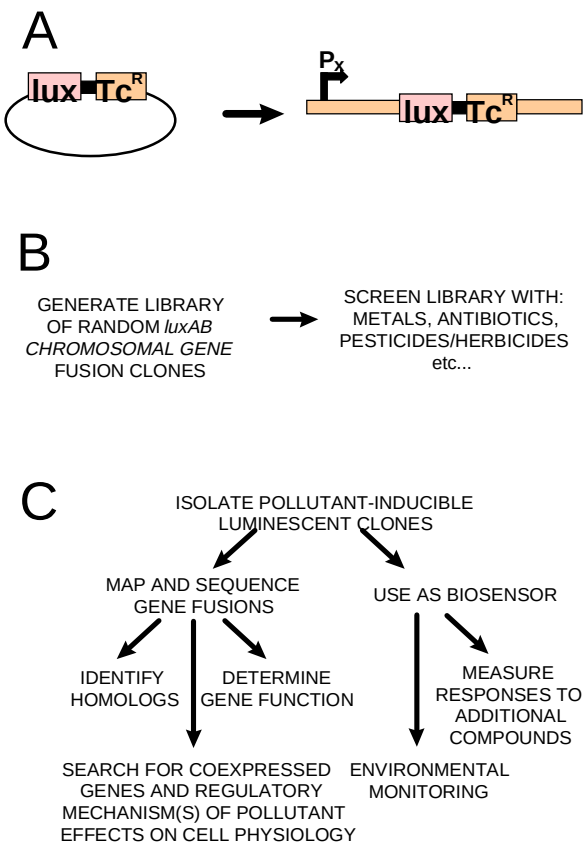

Fig. 2. Creation, isolation, and use of bacterial biosensors. (A), Depicted is the promoterless *luxAB* reporter gene (*lux*), a selectable antibiotic resistance marker (Tc^R) integrated into a random chromosomal location, downstream and under the transcriptional regulation of a promoter (Px). (B), First, a library of gene fusion clones, each with the promoterless *luxAB* transcriptional reporter fused at a single and random location in the bacterial chromosome is generated. Then, the library is exposed to metals, antibiotics, pesticides, herbicides, or any other compound of interest and light emission is monitored. Clones that consistently exhibit increased (or decreased) light emission upon exposure may be useful as biosensors. (C), Mapping and sequencing of the gene fusion allow identification of the gene responsible for the response. Homology searches determine conservation of this gene among prokaryotic and eukaryotic organisms. Identifying the function of the gene helps determine why it is expressed upon exposure to the compound of interest. Searching for coexpressed genes reveals the signal transduction pathway involved. The biosensor may also be tested for responses to additional compounds. Light emission from the ideal biosensor can be used to monitor, in real-time, bioavailable concentrations of a single (or a few closely related) compound(s) of interest.

biologically active forms will be biologically available [8, 10]. Organic and inorganic ligands, which are inevitably present in aquatic ecosystems, greatly expand the range of possible species and influence their biolavailability [8]. The *luxAB* gene fusion clones therefore represent powerful tools because they are living organisms which interact solely with bioavailable substances. Finally, the natural sensitivities of these biosensors have been tested by measuring the minimum and maximum detectable responses.

These clones also serve to aid in determination of the underlying physiology involved in the microorganism's response to the chemical. The identification of the gene, and its

associated regulatory cascade and signal transduction pathway, can yield important clues as to the nature of the genetically-programmed response(s) that has evolved to deal with the environmental contaminant. In addition, the DNA sequence of the *E. coli* chromosome has revealed the fact that approximately 40% of the genes have no known function ("orphans") that can be inferred by bioinformatics. As several of our clones have insertions within these unknown genes, this suggests that the study of these pathways will yield the identification of new gene and protein motifs that can then be added to the databases to help in the identification of similar genes in other organisms. Moreover, the behavior of these genes and their evolutionary conservation can also aid in identifying and predicting trends in the ecology of microorganisms in contaminated environments, as outlined in the next section.

The evolution of genetically-programmed responses to arsenic oxyanions

Arsenic is a ubiquitous metal that is also used in the common wood preservative, CCA (Chromated Copper Arsenate). In this process, called "Wolmanizing", CCA is pressurized into wood in order to prevent biotic attack and degradation. This type of wood product can be readily identified by its greenish tint. We have previously found that our *ars*B::*lac*Z or *ars*B::*lux*AB gene fusion biosensors can detect the presence of arsenic in CCA in a concentration-dependent manner [3]. We have also found that an *E. coli ars* mutant is over 10-fold more sensitive to arsenic oxyanions than the wild-type strain. In addition, we found that if the *ars* operon is cloned on a multi-copy plasmid (such as pBR322), *E. coli* is then over 100-fold more resistant (compared with the wild-type strain) to the same arsenic oxyanions. Lastly, we found that the *ars* operon is conserved in the chromosomes of a wide variety of eubacterial species [4, 7].

These results suggest several predictions for the ecology (and genetics) of microorganisms in the presence of high concentrations of arsenic (Fig. 3). In untreated

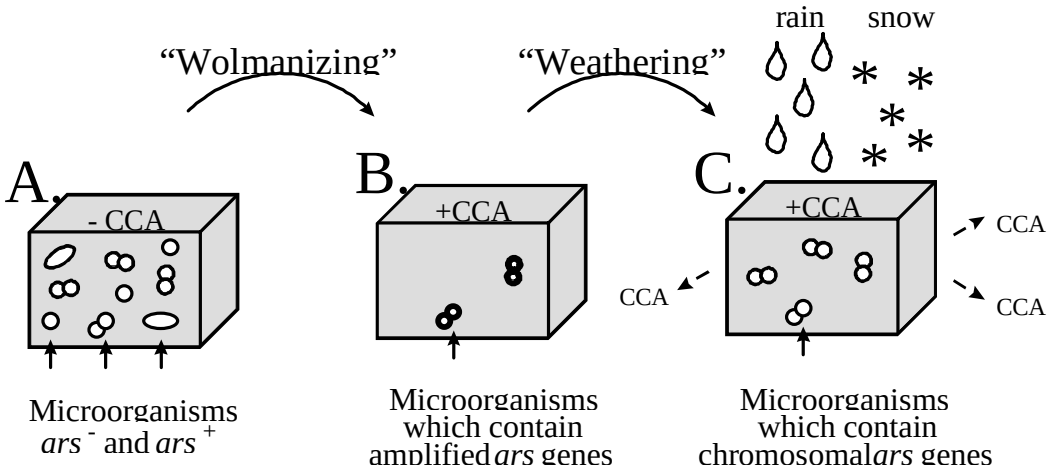

Fig. 3. The presence of microorganisms in untreated and CCA-treated wood. (A), Untreated wood showing the presence of different microorganisms with or without a gene conferring resistance to arsenic, denoted as *ars*$^+$ and *ars*$^-$, respectively. (B), Wood treated with CCA illustrating the survival of microorganisms which contain elevated copies of the arsenic resistance genes (*ars*). (C), Upon weathering and CCA leaching, wood treated with CCA shows the presence of microorganisms adapted to their environment (i.e. to a higher concentration of CCA). These microorganisms may contain single copies of the *ars* operon in their chromosome.

wood, a variety of microorganisms can be found. However, after Wolmanizing, only bacteria that are inherently resistant to arsenic (plus copper and chromate), or those containing amplified copies of the *ars* operon, should be found on the wood. These organisms will predominate due to the stochastic localization of the *ars* operon on a multi-copy plasmid. These selection pressures may also ultimately lead to the transmission of these plasmid-based *ars* operons to other organisms and thus to their proliferation to members of the same or different bacterial species. Through this combination of vertical and horizontal transfer of the plasmid and its subsequent evolution, stable plasmid-based *ars* operons should (and are) found in bacteria. These results cannot distinguish whether the ubiquitous presence of chromosomal *ars* operons is due to the early spread and integration of plasmid-based genes, or their presence in early life forms and subsequent amplification on plasmids in arsenic contaminated environments. In either case, selective pressure will lead to rapid evolution and transfer of these genes, a result also seen with the increasing use of antibiotics and the concommitant rise in plasmid-based antibiotic resistance genes. Nonetheless, the use of oligonucleotide probes now allows the detection and quantification of these genes in the absence and presence of selection and the determination of the relative contributions of endogenous gene amplification versus horizontal gene transfer to the microbial ecology of highly selective environments.

Conclusions

The luciferase transposon system has been successfully used to generate gene fusions which respond to environmentally important compounds. Clones developed in our laboratory identify genetically-programmed responses in bacteria for arsenic, selenium, aluminum, iron, and tributyl tin. The increase in light emission is readily measurable with film, a luminometer, and in some cases, the naked eye. Although we have only tested our system with *E. coli*, it should also function well in *Salmonella* and other *Enterobacteriacea* spp. Modification of the transposon delivery system, and substitution of the antibiotic resistance gene with other markers would allow use of this system in a broad range of prokayotes. Indeed, a diverse array of luciferase transposons have been developed and tested as gene fusion vehicles.

Bacterial biosensors are a tool with immense potential. Analytical devices and techniques are quite capable of identifying and quantifying compounds that are present in the environnment. However, biosensors have the advantage that they only respond to compounds that are present in biologically-relevant forms, and at bioavailable concentrations. The Microtox™, Biotox™, and Ecotox™ systems have used living organisms to evaluate water quality. However, these assays only detect compounds that are capable of killing or mutagenizing the test organisms. Moreover, they only indicate the presence, and not the identity, of the toxic agents. An appropriate battery of luciferase biosensors will be able to identify both the presence and identity of bioavailable compounds, and will do so even when these compounds are present at sub-lethal concentrations. Lastly, these clones provide important information for the identification and characterization of the underlying genetic and biochemical responses of microorganisms to these chemicals, and thus represent necessary tools for the elucidation of mechanisms that play a role in microbial ecology.

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