Bacterial volatile sensing

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Introduction

Quality control becomes an important aspect in modern life. We are becoming more and more aware about contents, quality, and security of products that we use to support our daily activities. Industrial processes, medical tests and environmental protection have also a high demand on this aspect. Industries need to ensure the quality of their products, the medical tests need to keep their functionality to give valid and dependable results and the environmental protection efforts need to prevent hazardous substances from polluting the environment. Sensing techniques are developed to accommodate those by detecting the presence of wanted and unwanted substances on the respective place/product. They come with different methods, principles, and outputs but in the future we want to have simple, rapid, continuous, sensitive and/or flexible sensors.

One of the available sensing techniques is using living microbial cells to detect and respond to a signal. These microbial-based sensors (MBS) work by using the whole microbial cell or a group of cells, detecting a signal from the environment and giving a response immediately after. This sensing mechanism was adopted from the natural response of bacteria to the environmental changes. The sensor and the response can be engineered to fit the sensing purposes. It is relatively easy to design and redesign the genetic circuitry for sensing and producing a robust signal (Tecon, *et al*., 2008) and also a sensitivity, i.e being able to detect very low amounts of impulse (Werlen, *et al*., 2003). Bacteria sense and respond to a broad range of stimuli. These include temperature or pH changes, nutritional starvation, stresses from external or internal sources, new food sources, toxins, metals, and also quorum signals. The sensing mechanisms for these environmental changes are as varied as the stimuli, and in this essay we will focus on the bacterial ability to sense chemical changes in the environment.

At first, the sensing mechanism was employed to detect the presence and amount of organic compounds, such as naphthalene, benzene, toluene, ethylbenzene, and xylene (King, *et al*., 1990; Sticher, *et al*., 1997; Ikariyama, *et al*., 1997; Willardson, *et al*., 1998; Applegate, *et al*., 1998) as one of the major contributors of environmental pollution. Bacteria have the ability to recognize and use organic molecules present in the environment as a source of carbon and because of that, a whole-cell biosensor for these organic pollutants can be engineered by placing the expression of a reporter gene under the control of a particular transcriptional activator

involved in their metabolic pathway (Daunert, *et al*., 2000). The biosensors for detecting the organic pollutants mentioned before were mostly applied in aqueous samples. This has some obvious advantages, such as ample supply of nutrients to the cells and ease of sampling. Unfortunately, the detection limit of most biosensors applied in aqueous samples is insufficient to detect pollutants at low, but environmentally relevant concentrations, because the target analyte has to be transported from the bulk solute to the cell and the process of transport in aqueous solutions is not necessarily fast (Werlen, *et al*., 2003). However, organic pollutants with low levels of water solubility often have significant gas-water partitioning coefficient, which in principle makes it possible to detect such compound in the gas phase (volatile) rather than the aqueous phase (Werlen, *et al*., 2003; Sandhu, *et al*., 2007). This way the sensitivity of the detection can be improved and the range of detection can be expanded. In this essay, I will focus on the advantages of volatile sensing with bacterial biosensors.

Bacterial adaptation

Bacteria have evolved a multitude of chemical mechanisms to detect changes in their surroundings, adapt their metabolism in response to scarcity or excess resources, and alter their pathogenic lifestyle according to the host (Salis, *et al*., 2009). This form of adaptation has provided competitive advantages towards bacteria or groups of bacteria over their competitors in their ecosystem. The current diversity of bacteria is impressive, especially with their ability to adapt under extreme environmental conditions such as diverse chemical composition, ranging pH, temperature and pressure. On the other hand there are many bacterial species have become symbionts or pathogens and have adapted to live in close proximity with other organisms (Arber, *et al*., 2000).

The bacterial evolution in adaptation is powered by many factors such as plasticity of the genome, the rate of phenotype generation, as well as selective pressure exerted by the environment (Arber, *et al*., 2000). In eukaryotes, genetic variability is primarily the result of sexual reproduction (chromosomal recombination during meiosis) while in prokaryotes (bacteria) this form of genetic variation is not available. The rate of evolution in prokaryotes is determined by frequent occurrence of point mutations, high level of recombination and gene

silencing, and the transfer of genetic material between different bacterial species—or even genera (Hacker, *et al*., 2001). With these abilities bacteria can survive by interacting with their environment and each other, adjusting and sharing information by genetic material to obtain phenotypes that are suitable to the environment, while the other competitors are diminished by survival selection. There are several mechanisms and/or combinations by which bacteria can adapt to the environment. First, there can be an increase in population size of bacteria to tolerate or degrade the compound by the induction of appropriate corresponding genes. The cells then can adapt through mutations, such as single nucleotide changes or DNA rearrangements that result in resistance to or degradation of the compound. Through all the possible processes mentioned, the bacteria may also acquire genetic information from either related or phylogenetically distinct populations in the community by horizontal gene transfer (Top, *et al*., 2003). The transfer of genetic material between different bacterial species/genera, referred to as horizontal gene transfer (HGT), represents a cornerstone of bacterial evolution, and it has led to dramatic changes on the composition of microbial genomes over relatively short time periods (Ochman *et al*., 2000) compared to point mutations, gene recombination and gene silencing, which have undergone a long evolution and are now fine-tuned for their present function (Arber, *et al*., 2000).

Bacterial gene transfer may happen between different closely related or distinct bacteria via conjugation, transduction and natural transformation depending on the bacteria. Not all part of the genomic DNA from one bacterium is able to be 'shared' or transferred to other bacteria. The majority of horizontally transferred DNA is part of flexible bacterial gene pool comprised of plasmids, conjugative transposons, simple transposons, integrons, genomic islets (<10 kb), and genomic islands (>10 kb). Different from the core gene pool which contains genes encoding proteins that play roles in basic cellular functions (translation, metabolism, cell architecture), the flexible gene pool encodes additional functions that are not essential for bacterial growth, but provide advantages under particular growth conditions (Hacker, *et al*., 2001). The horizontal transfer of genetic materials such as plasmids and transposons, often regarded as mobile genetic elements (MGEs), has been the generally accepted reason for the emerging environment resistance bacteria. For example, the emergence of antibiotic resistance bacteria has been considered as the result of widespread use of antibiotics and the release of large quantities of antibiotic compounds into the ecosystem (Davies, *et al*., 1994). The constant exposure of certain

bacteria or groups of bacteria to antibiotics can suppress the majority of the cells, but the slightly tolerant cells survive and develop resistance via various mutations. The surviving resistant cells flourish in the environment, carrying the resistant gene in their gene pool. If the genetic codes are stored inside the flexible gene pool, they can be transferred into another bacterium via HGT to become the part of its own genetic pool. If the receiving bacterium is closely related to the donor, the transferred gene has a high probability to be transcribed, translated properly and integrated into the cellular system.

Bacterial sensing mechanisms

To be able to adapt, bacteria have to sense the changes in the environment and give a response accordingly. The sensing and response mechanisms have to be synchronized so that the bacteria can immediately respond after receiving a signal / impulse from the environment. This sense – respond mechanism is coded inside the bacterial genomic DNA, as part of the evolutionary history of bacteria. In the previous section about adaptation it is stated that a bacterium can transfer its genetic material into another bacterium, for different purposes. This means that the genetic materials encoding sense - respond mechanisms are possibly transferable between bacteria. Moreover, using genetic engineering techniques, we can combine genetic codes of different sensing mechanisms with different responses to get the desired sense – response combination. In the emerging field of synthetic biology, a central goal is to reliably engineer bacteria to respond to environmental signals according to a genetic program (Andrianantoandro, *et al*., 2006).

In order to respond to a signal from their surrounding, bacteria or groups of bacteria utilize sensors that react specifically to the presence of certain environmental signals. Bacteria are found in almost all environments on earth, so the diversity of the sensors and signals is very high. This means that bacteria can detect and respond to almost every impulse in all environments. By rewiring the genetic components of the sensors and response/reporter together into new combinations, we can create novel sensor systems that respond to environmental signals that can benefit humanity in many ways (Voigt, *et al*., 2006). The usage of bacteria as chemical detectors started more than 20 years ago (King, *et al*., 1990) and has expanded since. The

bacteria were used as a whole cell biological sensor (biosensor) to detect specific chemical compounds and to give a measureable response, a reporter, such as bioluminescent protein (King, *et al*., 1990; Ikariyama, *et al*., 1997; Sticher, *et al*., 1997; Werlen, *et al*., 2003; Vijayaraghavan, *et al*., 2006). The concept of bacterial sensing is to have a circuitry of sensor-reporter mechanisms: the reporter protein is to be produced by the cell after specific contact or interaction with a target analyte and/or condition. In principle, this sensor-reporter mechanism mimics the reaction of cell from sensing the signal from the environment to the production of proteins as a reporter. In most of the current designs, the synthesis of the reporter protein is under control of a transcription factor which directs the repression or induction of reporter gene expression from a dedicated site on the DNA (e.g. promoter) (Tecon, *et al*., 2008) and the sensory function can be provided by the transcription factor itself via an internal effector binding domain that transmits target perception to form interactions with RNA polymerase (van der Meer, *et al*., 2004) or via a sensory protein (Fig.1).

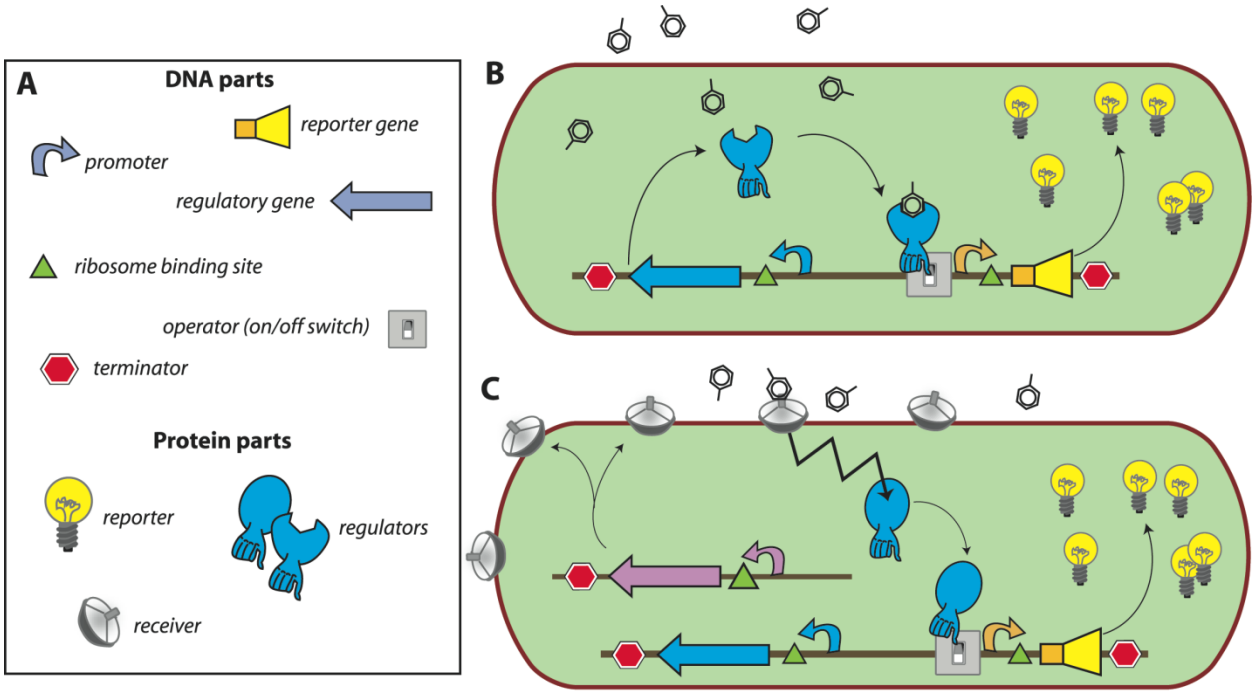

Fig.1. Bacterial sensor – reporter cell (Tecon, *et al*., 2008).

(a) The DNA parts necessary for construction a circuitry of sensor – reporter function. These parts can be assembled by genetic engineering techniques. Regulatory genes are for the sensing function and the reporter genes are for the output of the system.

(b) A setup in which the sensor function is provided by a single regulatory protein. The regulator protein binds the target compound and induces the transcription of the reporter gene, leading to the production of reporter proteins.

(c) A setup in which the sensor is separated with the regulator function. The target compound is sensed by a periplasmic receiver protein that transmits the detection event via a signaling cascade to the regulatory protein (the zigzag arrow). The activated regulator then induces reporter gene leading to the production of reporter proteins.

The specificity of target detection is determined by recognition specificity of the sensors and other condition that influencing the signaling cascade or acting on the same promoter (Marqués, *et al*., 2006). When the signal from the environment is received by the sensor, the transcription of reporter genes is induced and production of reporter proteins started. The communication between the sensing system and the reporter system is very important. It could be just a simple induction or a cascade of signals to the regulatory protein regulating the reporter gene. The sensing event can be measured after translation and amplification into protein reporter synthesis, which in this case the amount of reporter protein produced determine to the amount of the impulse sensed.

On top of all the sensing and response mechanisms, in order for a system to be working properly, bacteria need to be in some sort of active state in an environment providing enough resources to produce the required response (Tecon, *et al*., 2008). This might pose a serious problem for this system's shelf life, but on the other hand it is embedded in self propagating entities that are cheap and easy to produce. The fact that bacteria can be engineered to have different sensors with the same or different reporters, or the other way around, gives us a wide range of selection and possible combinations to create our own specific sense-respond systems.

Bacterial biosensors

A bacterial biosensor is a sensing tool of bacteria to detect changes from the environment. There are two different environments that affect bacteria, the intracellular environment and the extracellular environment. Intracellular environment is an area inside the bacterial cell itself

consisting of cell organelles and bio-macromolecules, such as proteins, DNA and RNA, while extracellular environment is the area surrounding the cell and all that affects its condition. Bacterial biosensors in both environments are needed for various purposes in order for bacteria to survive. Both types of sensors have some differences and similarities in their working mechanism, but they have the same purpose: to work as a sensing tool. This chapter will be focused on example of biosensors used in intracellular and extracellular sensing.

I. Intracellular biosensors: Allosteric transcription factors

Allosteric transcription factors are the most commonly studied type of bacterial sensor. When bound to cytoplasmic small molecules, these proteins will undergo an induced conformational change that alters their DNA binding specificity, enabling the targeted regulation of gene transcription (Salis, *et al*., 2009). This sensor is often found to be responsible to regulate the expression of transporters and enzymes that import and break down specific nutritional sources, for example LacI for allolactose (Fig.2), MalT for maltose, and AraC for arabinose (Kaplan, *et al*., 2008). This sensor was also found to react to toxins, such as antibiotics, by activating the expression of efflux pumps or other proteins that nullify the toxin's effects. There are several roles of allosteric transcription factors in cellular metabolism, such as the transcription factors Crp and Fnr (Körner, *et al*., 2003), which sense the availability of ATP and respiratory potential by respectively binding to cyclic AMP and molecular oxygen. The Crp transcription regulator upregulates the genes involved in carbon utilization and energy production when there is an accumulation of cyclic AMP. The Fnr transcription factor regulates the key genes responsible for aerobic and anaerobic metabolism in response to the presence or absence of oxygen. This transcription factors can regulate the same promoter to control the rate of transcription. This creates a simple logical 'decision-making' for the cell in regulation and fine-tuning the expression level of the promoters. For example, AraC and Crp are transcription factors that regulate the operon for arabinose utilization. The activation can only occur when the two transcription factors are activated, which is when there is a low ATP concentration and sufficient arabinose present (Kaplan, *et al*., 2008).

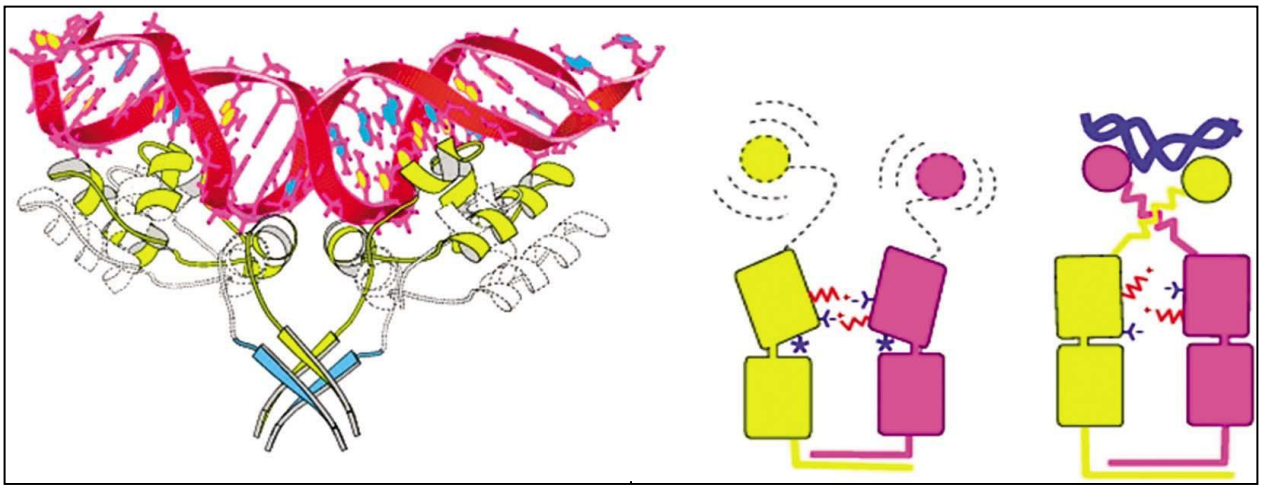

Fig.2. An example of an allosteric transcription factor, the *lac* repressor. The repressor is active when there are no allolactose or IPTG (the analog of allolactose used in lab experiments) present. The left figure explains changes in the DNA-binding domains of *lac* repressor from the induced to the repressed conformation. The DNA complex is shown in yellow and the IPTG complex is shown in blue. The middle and right figures are simplified cartoon figures summarizing the allosteric changes of *lac* repressor. The circles are DNA-binding domains connected to the squares, which are dimers of regulatory domains, via linkers (sometimes called hinge helices). The middle figure shows dimers of the *lac* repressor bound to IPTG (asterisk). The addition of IPTG causes changes in the NH_2-terminal subdomains of the dimers, which causes the hinge helices in repressor to move apart. This movement disrupts the dimerization of the helices and the helices become disordered. A number of salt bridges exist between the N-terminal sub-domains of the core, and the N-terminal sub-domains are rotated and translated apart compared to the DNA-bound form. The right figure shows a dimer of the *lac* repressor-DNA complex. The DNA is represented by the purple double coil. The salt bridges that exist in the IPTG-protein complex are broken by the movement of the N-terminal sub-domains of the core. The hinge helices are present in the DNA-protein form. This figure is adapted from Lewis, *et al*., 2005.

II. Intracellular biosensor: Riboswitches

Riboswitches are RNA-based sensors that form mRNA structures capable of switching their conformation from one state to another (Fig.3) in response to the intracellular environmental changes (Vitreschak, *et al*., 2004). This mRNA sensor is shown to regulate several metabolic pathways involved in the biosynthesis of vitamins, amino acids and purines. Riboswitch conformational changes are induced by the presence of certain small molecules/metabolites or through variations in temperature leading to the modulation of gene expression, including transcriptional termination, translational initiation, and mRNA stability (Salis, *et al*., 2009). In most cases one of the alternative structures, for example the repressing conformation, contains a terminator of transcription or a paired region covering the translation initiation site, whereas in the other alternative structures (the non-repressing conformation) this regulatory part of the structure is destroyed and the gene or genes are expressed. For example, a riboswitch in the *ydhL* mRNA of *Bacilus subtilis* responds to the absence of adenine by forming an early transcriptional terminator, lowering the production of adenine efflux pumps (Zakataeva, *et al*., 2007). A short region in the mRNA (aptamer) binds to adenine molecules and forms a tertiary structure. The formation of long RNA hairpin in this structure is responsible for transcription termination. When adenine is no longer present, the new mRNA transcripts no longer form tertiary structures allowing early transcriptional termination to occur. Riboswitches also play an important role in termperature sensing such as RNA thermosensors that regulate translation initiation depending on the temperature changes (El-Samad, *et al*., 2004).

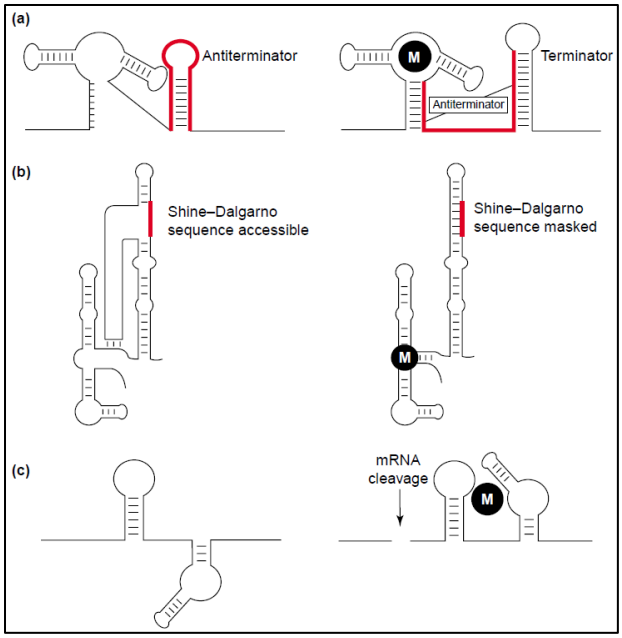

Fig. 3. Mechanisms of a riboswitch function. (a) Transcription termination induced by small molecules/metabolite (M) binding to nascent RNA, as observed for a guanine riboswitch. (b) Translation initiation modulated by metabolite-dependent sequestration of a Shine-Dalgarno sequence, as observed for a TPP riboswitch. (c) RNA processing regulated by metabolite-dependent self cleavage. This figure is adapted from Soukup, *et al*., 2004.

III. Intracellular biosensors: stress and starvation biosensors

In order to survive, bacteria have to quickly adapt to hazardous environments by sensing a variety of stressful physical and/or chemical stimuli and regulate their gene expression to compensate the damage. There are TCSs that work in favor of hazardous physical stimuli, for example, the TCSs Rst and Cpx can detect instabilities in the inner membrane caused by the excess unfolded protein and then triggering the σ^E-mediated envelope stress response, which activates the expression of a variety of periplasmic chaperones and proteases (Rhodius, *et al*., 2006). Despite the mentioned sensors before, there are special sensors that are sometimes required to detect certain stress stimuli, for example starvation biosensors and presence of reactive oxygen species.

The ribosome-bound RelA kinase is responsible for detecting amino acid starvation (Traxler, *et al*., 2008). When an uncharged tRNA binds to the ribosomal A-site, the RelA kinase

synthesizes one molecule of ppGpp or pppGpp. When free amino acids become depleted, the corresponding tRNA remains uncharged, causing an accumulation of ppGpp or pppGpp and triggering a transcriptional regulation called the stringent response. The ppGpp can destabilize the open complex formation of RNA polymerase at a promoter. Promoters with weak open complex formation are further destabilized causing transcriptional repression, while promoters with strong open complex formation are unaffected / activated by increasing the amount of free RNA polymerase. The entire cellular metabolism is shifted to maintain a set of biomass-protein ratio. The amino acid biosynthetic pathways are activated while the ribosome, nucleotide, and fatty acid synthesis pathways are repressed.

Reactive oxygen species such as singlet oxygen ($O_2\,{}^1\Delta_g$), superoxide (O_2^-), and hydrogen peroxide (H_2O_2) initiate massive cellular damage by oxidizing chemical groups or disrupting the iron-sulfur clusters inside enzymes. Bacterial sensors must detect reactive oxygen species and rapidly trigger the production of antioxidants and enzymes that convert them into their non-toxic forms. In *E. coli*, transcription factor SoxR is induced by superoxide and activates the SoxS regulon, which includes superoxide dismutase, SodA (Gaudu, *et al*., 1996). Hydrogenperoxide induces the OxyR factor, activating the expression of the hydrogenperoxide decomposer enzyme, the catalase KatG (Blanchard, 2007).

IV. Extracellular biosensor: Two-component systems

Lacking a transmembrane component, both allosteric transcription factor and riboswitches are limited to sensing only intracellular environmental changes. A two-component system (TCS) is a transmembrane sensor kinase that biochemically transduces a signal to a cytoplasmic response regulator, which binds to DNA to regulate transcriptional initiation (Salis, *et al*., 2009; Mascher, *et al*., 2006). The sensor kinase is responsible for sensing the environmental changes and responding by phosphorylating its cognate response regulator. The phosphorylated response regulator increases its DNA binding specificity and regulates the rate of transcriptional initiation (Fig.4). Sensor kinases often bind to the periplasmic membrane as dimers or larger multimers. All of these traits, the domain structure of the sensor kinase, the DNA binding properties of the response regulator, and the number and positioning of the DNA operators inside the promoter provide a path from stimulus to gene regulation. Manipulating

these traits can lead to new sensors that respond to different signals or promoters that respond to different response regulators (Salis, *et al*., 2009).

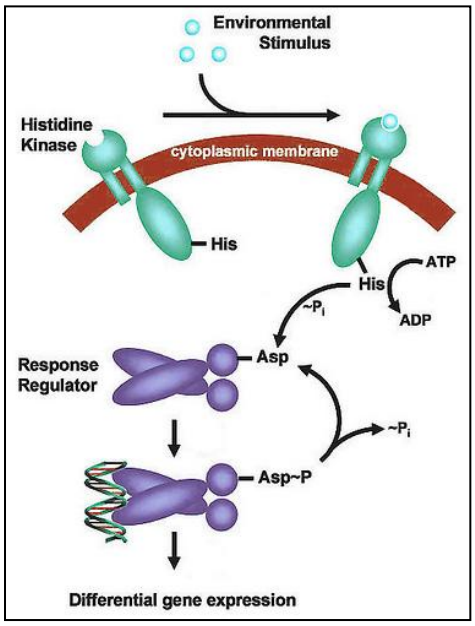

Fig. 4. General mechanism of the two-component system in bacteria. The sensor kinases often bind to the periplasmic membrane for sensing the environmental changes and responding by phosphorylating its cognate response regulator. The response regulator then increases its DNA binding specificity and regulates the rate of transcriptional initiation.

Table 1. A table of several examples of two-component systems, with the stimulus and the effects on their response regulator, along with the regulated promoters (SK = Sensor Kinase, RR = Response Regulator).

TCS names SK -> RR	Stimulus and Effect on RR	Example of promoters (RR binding sites)	Reference(s)
Metabolic responses			
ArcB -> ArcA	low oxygen increases ArcA-P	fadB, ssb	Liu, *et al.*, 2004; Cho, *et al.*, 2006
AtoS -> AtoC	high acetoacetate increases AtoC-P	atoD	Grigoroudis, *et al.*, 2007; Matta, *et al.*, 2007.
NarQ -> NarP	high nitrate and nitrite increases NarL-P and NarP-P	nirB, fdnG	Wang, *et al.*, 2003; Partridge, *et al.*, 2008.

NarX -> NarL	high nitrate increases NarL-P	fdnG focA	Wang, *et al.*, 2003; Partridge, *et al.*, 2008.
Metal/ion responses			
CusS -> CusR	excess copper increases CusR-P	cusR, cusC	Yamamoto, *et al.*, 2005;
PhoR -> PhoB	low external phosphate increases PhoB-P	phoA, ugpB	Blanco, *et al.*, 2002.
Stress responses			
CpxA -> CpxR	unfolded perisplamic protein	degP, ppiD	de Wulf, *et al.*, 2002; Batchelor, *et al.*, 2005.
RstB -> RstA	stress caused by low pH or phosphate, increases RstA-P	asr, csgD	Ogasawara, *et al.*, 2007.
RcsCF -> RcsD -> RcsAB	low temperature, high glucose, or high zinc increases RcsAB-P	flhD, ftsA	Hagiwara, *et al.*, 2003; Vianney, *et al.*, 2005.

In TCS, the sensing and response communication is provided by protein to protein interactions by activating or repressing gene expression according to a single stimulus from the environment. Similar to allosteric transcription factors, multiple response regulators in TCS can also bind to a single promoter, creating a logical choice mechanism, controlling the rate of gene expression that uses multiple stimuli as inputs. An example is the FocA formate transporter. It is activated by the ArcAB TCS under anaerobic conditions and repressed by NarXL TCS in response to high nitrate availability (Kaiser, *et al*., 1994; Kaiser, *et* al., 1997). The *focA* promoter also binds to Crp and Fnr transcription factors (mentioned before) to integrate a total of four oxygen and energy-related (ATP) signals as inputs into its genetic system.

V. Extracellular biosensors: Quorum sensors

A quorum sensor is a sensor system that detects and responds to a diffusible molecule that is produced by a population of organisms, frequently from multiple species (Salis, *et al*., 2009). The diffusible molecule is also called autoinducer, it dynamically changes according to multiple factors, which are the production rate of the autoinducer, the number of organisms producing the autoinducer, and volume of the surrounding environmental space (Waters, *et al*.,

2005). Quorum sensing bacteria produce and release chemical molecules as signals to the environment whose external concentration increases as a function of increasing cell population density. Bacteria can detect the accumulation of a minimal / threshold for stimulatory concentration of this autoinducer molecules and alter gene expression as a response. In Gram negative bacteria, there is a prototypical architecture of a quorum sensing system, consisting of an autoinducer synthetase that produces a diffusible, membreane-permeable homoserine lactone (HSL) and an allosteric transcription factor that binds to it (Salis, *et al*., 2009). This architecture is named LuxIR type after the first such quorum sensor system discovered in the marine bacterium *Vibrio fischeri*. In Gram positive bacteria, the autoinducer is a secreted short peptide that binds to membrane-bound sensor histidine kinases as sensors from the TCS. The signaling is then mediated by a phosphorylation cascade that influences the activity of a DNA-binding transcriptional regulatory protein termed a response regulator (Waters, *et al*., 2005). A quorum sensoring system in general is a combination between TCS and allosteric transcription factors. The quorum signals are not directly involved in any single environmental stimulus, but rather involve the cell to cell communication. Although the response is not directly into any environmental stimulus, the goal of this cell to cell communication in many bacteria is to survive by forming multicellular surface-bound aggregates, or biofilms, whose remarkable feats of persistence are the problem of both medicine and industry (Nadell, *et al*., 2008). In an ecosystem of microbes, each species can regulate productions of quorum signals in response of different stimuli. The mixing of the signals, the concentration of the autoinducers and their effects on gene regulations depend on the summation of all the signals and in the proportion of the sending organisms (Salis, *et al*., 2009).

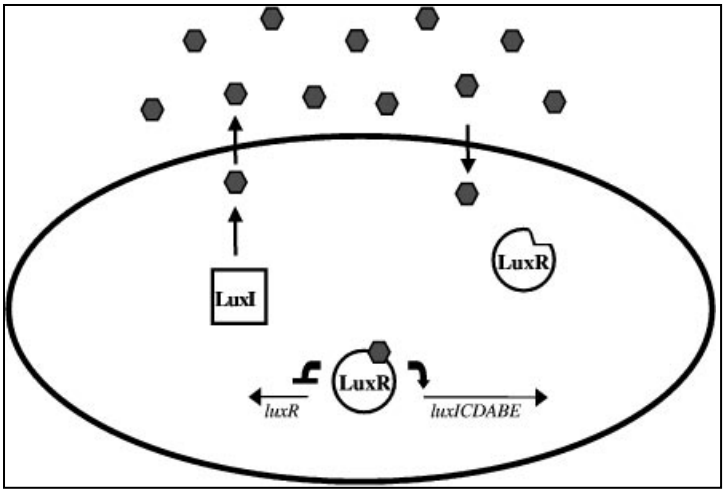

Fig. 5. The *Vibrio fischeri* lux quorum sensing circuit, consisting of five luciferase genes (*luxCDABE*) and two regulatory genes (*luxI* and *luxR*). The LuxR-autoinducer (the autoinducer is hexagonal shaped) complex binds to both the left and right promoters of the lux system. This represses expression of *luxR* and activates expression of *luxICDABE* genes respectively. The result is an exponential increase in autoinducer, through increased expression of *luxI* and an exponential increase in light emission through the increased transcription of LuxCDABE. This figure is adapted from Miller, *et al*., 2001.

Reporters for cell-based biosensing

Expression of a reporter gene produces a measureable signal, which can be readily distinguished over the background of endogenous proteins in the environment. For analytical uses, reporter genes or their corresponding proteins are often coupled to a sensing element which recognizes an analyte and thus confers selectivity to the system while the reporter protein produces a detectable signal, determining the system's sensitivity (Wood, *et al*., 1995). There are several characteristics required for a useful reporter gene. First, the quantification of the reporter-gene expression or activity must be conducted using a simple assay. Second, the amount of reporter protein activity must reflect the amount of the analyte sensed by the sensors. Finally the reporter protein must be easily distinguished over the background proteins, in order for easy quantification. Several unique reporter proteins have been employed in cell-based biosensing systems, namely, chloramphenicol acetyltransferase, β-galactosidase, bacterial luciferase, firefly luciferase, aquorin, green fluorescent protein, and uroporphyrinogen III methyltransferase

(Daunert, *et al*., 2000). There are advantages and disadvantages for each of these mentioned reporters based on the assay conditions and their detection method (Table 2). The choice of reporter is dependent upon the background endogenous activity of the cell line used, gene expression and transfection efficiency, and the detection method as well as the analytical application system (Wood, *et al*., 1995). The mentioned reporters can be measured quantitatively in a facility harboring a measuring device for the corresponding reporters. For ease of use, a semi-quantitative and/or qualitative reporter such as colour pigments can also be used as detection tests outside the facility that need immediate result without any quantifying facility. The choice of reporters should be made by referring back to the criteria of a good reporter, the compatibility of the reporter gene and activity in the bacterial host and the ease of measurement for the reporter's activity.

Table 2. Advantages and disadvantages of reporter proteins used in whole-cell biosensing system (Daunert, *et al*., 2000).

Reporter protein	Advantages	Disadvantages
chloramphenicol acetyltransferase	No endogenous activity.	Often employs radioisotopes. Requires addition of a substrate. Requires separation of substrate and product. Narrow linear range.
β-galactosidase	Sensitive and stable. Moderate linear range. Applicable in anaerobic environment.	Endogenous activity. Requires addition of a substrate.
bacterial luciferase	High sensitivity. Does not require addition of a substrate. No endogenous activity in mammalian cells.	Heat labile therefore limited use in mammalian cells. Narrow linear range.
firefly luciferase	High sensitivity. Broad linear range. No endogenous activity in mammalian cells.	Requires addition of a substrate. Requires an aerobic environment and ATP.
aequorin	High sensitivity. No endogenous activity in mammalian cells.	Requires addition of a substrate and the presence of Ca2+.
green fluorescent protein	Autofluorescent, therefore, does not require addition of a substrate or cofactors. Spectral variants. No endogenous homologues in most systems.	Moderate sensitivity. Requires posttranslational modification. Background fluorescence from biological systems may interfere. Potential cytotoxicity in some cell types.

	Stable at biological pH.	
uroporphyrinogen III methyltransferase	Autofluorescent, therefore, does not require addition of a substrate or cofactors. May have better signal-to-noise ratio than green fluorescent protein. Does not require costly reagents or special host strains.	Endogenous activity.

Application of whole-cell biosensors

Whole cell biosensors have been used for chemical sensing in aqueous solution or slurries of soil-water to detect specific chemical compounds / analytes contained inside. For example, the bioluminescent reporter *Pseudomonas fluorescens* strain HK44 that carries a transcriptional *nahG-luxCDABE* fusion for naphthalene and salicylate catabolism was used to assay the bioavailbility of naphthalene and salicylate quantitatively in soil slurries (Heitzer, *et al*., 1992). The bacteria themselves have the ability to degrade naphthalene into 2-oxo-4-hydroxy-pentanoate in two steps, first degradation of naphthalene into salicylate and then the salicylate is degraded into 2-oxo-4-hydroxy-pentanoate. The presence of salicylate induces the *nah* operon, increasing the expression of *nahG* leading to the increased production of bioluminescent protein from expressed *luxCDABE* genes. Another example is the engineering of a whole cell biosensor of linear alkanes by utilizing the *alk* regulon induced by the presence of alkanes and coupling it with a bacterial luciferase reporter, under regulation of the induced operon (Sticher, *et al*., 2000). Plasmid pJAMA7 constructed with a fusion of the *alkB* promoter of *Pseudomonas oleovorans* and the *luxB* genes of *Vibrio harveyi* and plasmid pGEc74 containing *alkS*, a regulatory gene from *Pseudomonas oleovorans* to activate *alkB* promoter, were transformed into *E. coli* strain DH5α. In this system, the *alkB* promoter is activated by the transcriptional activator protein AlkS cloned into the host cell. When the cells sense octane in their surrounding, they emit bioluminescence light from the reaction of expressed luciferase. Elad, *et al*. (2008) developed the idea of a microbial array chip containing different genetically engineered bacteria to detect

various chemicals immobilized in a specific order on various polymers such as poly dimethyl-siloxane (PDMS). The microbial whole-cell array functions as a multi-detector of various chemicals that are present in environments. The engineered bacteria were kept alive on the chip and the aqueous sample from environmental was incubated in contact with the bacteria on the chip. The responses from the bacteria (cell size, number, viability, bioluminescence, fluorescence, etc) were recorded via corresponding detector.

The sensing system by diluting the analyte in aqueous samples proved to be working in most of the cases of detecting organic pollutants. Unfortunately, the detection limit of most biosensors is insufficient to detect pollutants at low but environmentally significant concentrations (Werlen, *et al*., 2003). The settings of the detection cannot deliver analytically useful signals at target compound concentrations below 0.5 to 1 µM. By using the gas phase, the bioavailability amount of the analyte to the biosensor cells can be increased, thereby effectively lowering the detectable analyte concentration range (Werlen, *et al*., 2003). In the experiment performed by Werlen¸ *et al*., (2003), the measurement of naphthalene in aqueous phase and gas phase were compared using genetically engineered *Pseudomonas putida* carrying a NAH7 plasmid and a chromosomally inserted gene fusion between the *sal* promoter and the *luxAB* genes. Using the same bacteria and the same amount of analyte with different physical contacts with the analyte, the luciferase activity exhibited by bacteria exposed to naphthalene in gas phase was higher compared to the bacteria exposed by the aqueous phase. The bacteria were also incubated in a vial with an increase of sample volume with the same amount of naphthalene in gas phase (thereby effectively reducing the naphthalene concentration) compared to the same bacterial cells incubated in 5-fold more concentrated naphthalene (less volume). The detection sensitivity from the gas phase outperformed the one from the aqueous phase. The development of gas phase detection was astonishing at that time because most organic compounds targeted by bacterial biosensors (BTEX, alkanes, naphtalenes, chloroethylene, and chlorophenol) have significant gas phase partitioning (Applegate, *et al*., 1998; Sticher, *et al*., 1997; Hay, *et al*., 2000).

Discussion and future possibilities

The use of genetically engineered whole cells containing reporter genes coupled to biological recognition components allows for the design of rapid, highly specific, and sensitive biosensing systems. A wide range of applications of this technology is currently under investigation in areas including biotechnology, pharmaceutical analysis, diagnostics, environmental monitoring, and household quality control. To date, there are huge genomic resources and databases of diversity within the bacterial world to be exploited for designing bacterial biosensors. As mentioned before, bacteria can live in almost all environmental conditions by sensing and responding to their surroundings. The sensing capabilities are imprinted in a bacterium's genomic DNA pool and can be re-wired to a specific response and/or to reporters to function as a detector for specific signal from the environment. In addition, the method detection limits, are often in nanomolar range (van der Meer, *et al*., 2004), thereby competing effectively with the existing chemical analytics. Cultivation of bacterial cells is easy and the production costs are very low. The ability of bacteria as biosensors are proven to have field robustness, good measurement precision and accuracy in comparison to chemical analytical methods, as in case of detection of arsenic in groundwater (Trang, *et al*., 2005) and in rice (Baumann, *et al*., 2007). However, to place present biosensors detection systems in comparison to the advanced analytical chemistry detection system is probably not the wisest and the fairest thing to do. The high end chemical analytics such as gas chromoatography-mass spectroscopy (GC-MS), high-performance liquid chromatography-mass spectrometry (HPLC-MS), inductively coupled plasma-mass spectrometry (ICP-MS), etc., is superior per se to biosensors in terms of sensitivity (due to high signal-to-noise ratios), quantification and identification of unknown molecular structures and bears only the disadvantage of requiring substantial financial investments (Harms, *et al*., 2006). In terms of limit of detection, there is hardly any substantial superiority of chemical analysis compared to the biosensing-bioreporters mechanism. Most bioreporter constructs detect nano- to micromolar concentration of target chemicals, e.g. concentration down to o.1 fM mercury were detected with a luminescent *Escherichia coli*-based bioreporter strain (Virta, *et al*., 1995).

This assay is focusing on the mechanisms of detecting chemicals from the environment via gas phase (volatiles). As mentioned previously, detection of chemicals in its gas phase sample is more accurate and effective compared to the aqueous phase sample. This opens up new

possibilities for detection, gas/volatiles detection. There are a lot of possible gas/volatiles detections, such as natural gas detection, toxic pollutant detection, and gas leak detection. The performance and detection limits of bacterial biosensors are comparable to the existing chemical analytics detection, with considerable advantages such as possible miniaturization, portability, and financial benefits. The idea is to find biosensors that are responsive to target volatiles and couple them to a reporter. There is one method developed by iGEM team Groningen 2012 using existing DNA microarray analysis technique to check the up-regulations and down-regulations of bacterial genes as a response of bacteria exposed to the target volatiles. These genes can be used as positive and negative response switch coupled with reporters to act as a sensing construct. Specific to the substrates, these biosensors could be studied more to reveal their actual role in the bacterial metabolism pathway and therefore can be exploited furthermore. While this idea is still in development, it brings possibilities of future volatiles detection method that can complement the existing chemical analytic techniques.

Most efforts of bioreporter development are invested into the genetic constructs rather than in optimization of detection strategies, leaving considerable room for improvement. The self-reproducing trait of bacteria also makes them potentially instable (prone to mutations) because they remain complex living entities. Even a simple thing as introduction of an extra copy of a promoter/operator DNA fragment fused to a reporter gene can alter and/or add new functions to an existing capability. Despite of the disadvantages, bioreporters can be used for spatially restricted environments to restrain the spreading of the GMO, it can be used as an alternative analytical technology in labs with smaller budgets or for simple applications, and it could be miniaturized easily, allowing potential rapid multi-target analysis. This technology is invaluable for applications such as on-site environmental monitoring, home testing systems, and specific target diagnostics. The higher sensitivity for use with small sample sizes and the selectivity for use provided by this system have already indicated their potential for miniaturization and portability. Combining this technology with advances in electronics, computer science, and advance materials will improve detection strategies and allowing a greater degree of sensitivity than currently available at ultralow levels of analyte.

Applications of whole-cell biosensors are still rarely seen outside research laboratories (Harms, *et al*., 2006). Biosensors/bioreporters have made an astounding career in research institutes and are generally considered as valuable tools for microbiological, toxicological, and

environmental research. With every new technology, there is a risk that its superiority over existing technologies is not sufficient for market / general implementation or that drawbacks associated with the new technology compensate its potential advantages. The possibilities of GMO release and/or natural performance of bacteria to acquire new competences in the environment and public acceptance restrict this technology to be used outside laboratories. For more than two decades the biosensors/bioreporters technology has been developed, but it seems that the research into cellular biosensors is still mostly in a proof-of-principle or demonstration phase, not close to extensive use or commercial use outside of academia. Safety is the main public concern in this field, but with proper information to the public and with help of the authorities, this technology might someday be put active outside the research facility. One can conclude that convincing reasons are needed to decentralized mass application of GMOs and it is up to society to decide whether biosensors/bioreporters applications bear enough advantages to outweigh the anticipated risks. From a technical point of view, many of whole-cell constructs are ready for environmental application.

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