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Using the stress response to monitor process control: pathways to more effective bioremediation

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Summary

The use of microbial stress response as a basis for process control of microbiological systems is examined in this primarily forward-looking review of the literature. Although microbial stress response has been the subject of intensive laboratory investigation, the environmental reflection of the laboratory response to specific stresses has been little explored. However, it is only within an environmental context, in which microorganisms are constantly exposed to multiple changing environmental stresses, that there will be full understanding of microbial adaptive resiliency. It is our opinion that this more holistic understanding will first be developed in areas of applied microbiology, in which microbial growth is selectively fostered or limited.

Introduction

The contamination of large areas of the environment with organic and inorganic pollutants has promoted research in the development of effective bioremediation strategies. There is often no alternative to the bioremediation option, since the physical removal of soil, sediments, and subsurface materials that may extend many hundreds of meters in area and depth is not feasible. The tremendous variety of pollutant types, including compounds such as halogenated organics that are recalcitrant to microbial degradation and others that cannot be degraded, such as heavy metals and radionuclides, impose site-specific requirements that often prevent the general application of information developed at one site being used to design treatment strategies for other systems and pollutant types. As a result, there has been an increasing emphasis placed on site monitoring – to insure that specific microbial processes are operative or alternatively that intrinsic processes are sufficient to either control (e.g., minimize migration) or ultimately mitigate the contamination. Existing monitoring tools attempt to develop clear links between pollutant transformation and microbial activity. Ideally, a stoichiometric balance would be achieved – the disappearance of the pollutant directly correlated with the appearance of a metabolite, such as CO₂ from pollutant mineralization or chloride ion release via microbial dehalogenation. Since complete stoichiometric balance is virtually impossible in the open environment, having an unknown subsurface composition that is sampled at relatively few sites, monitoring has also

included the use of stable isotopic measures to provide additional evidence of microbial attribution. Stable isotope probes (SIP) and compound-specific isotope analysis (CSIA) have been used to determine rates of substrate transformation and to link processes with specific populations. For example, SIP has been used to determine the rate of electron donor fractionation into microbial metabolites like CO₂ and CH₄ [1]. The contribution of specific populations has been determined by tracing incorporation of specific organic components (e.g. ¹³C labeled lactate) into diagnostic phospholipid fatty acids (PLFA) and nucleic acids [2,3]. CSIA has been used recently to determine rates of utilization of several electron acceptors, e.g., oxygen [4], hydrogen [2], sulfate [5], nitrate [4], carbon dioxide [1] and dehalorespiration of chlorinated solvents [6]. These techniques have been used both in situ and in bioreactors to provide more direct evidence of metabolic state, kinetics, and stress status [7]. Less specific measures include a demonstration of increased microbial abundance and activity in areas of pollutant disappearance or documenting that microorganisms possessing the desired metabolic characteristics are present at the bioremediation site and increase in numbers with increasing rates of pollutant loss.

These monitoring tools when combined (e.g., SIP and CSIA are now being used concomitantly with PLFA, nucleic acid, and protein analysis) offer a measure of process rates and some attribution to participating microbial populations. However, they are primarily retrospective measures that provide only a very blunt instrument for effective process control. We suggest that more effective process control will derive from the development of monitoring tools that more directly determine the physiological status of organisms participating in pollutant transformation, and use that information for more effective optimization of site conditions as needed to promote rapid and desired microbial transformations. Thus, this review primarily concerns the development of microbial stress response systems as an emerging tool for more effective and general process control of bioremediation and other processes mediated by complex microbial communities. This tool would build directly upon existing monitoring technology, since knowledge of the microbial populations active in a desired transformation would provide a framework for using stress response for more effective process control.

What is stress? Microorganisms have relatively few behavioral options for coping with constantly changing environmental conditions (Figure 1). Although motility and chemotaxis are

important for optimizing local physical/chemical environment, they do not provide protection from rapid system-wide changes. Since running away is generally not an option, the microbial alternative is rapid adaptation, often involving one or more systems of stress response. Although microorganisms in the open environment are often stressed, and simultaneously exposed to multiple stressors, the study of microbial stress response has been primarily restricted to laboratory systems, considering the response of well-characterized “model” organisms to very specific types of stress. This somewhat reductionist characterization of stress has resulted in a somewhat unsatisfying set of definitions of microbial stress, generally including the following:

- Any deviation from optimal growth conditions that results in reduced growth rate
- An environmental situation that results in damage of cellular components in the absence of a cellular response
- Any situation that stimulates expression of known stress-response genes

The laundry list of better-studied stressors and microbial response systems includes: starvation, heat shock, cold shock, envelope stress, oxidative stress, oxygen deprivation, osmotic challenges, acid stress, sodium stress, and SOS response to DNA damage. Stress is also relative – temperature and pH that are stressful to one species may be optimal for the growth of another. Thus, we suggest the need for a more environmentally relevant definition of stress, one that incorporates the concept of microbial niche and the adaptive landscape. The most generally accepted definition of niche is that proposed by Hutchinson [8] – “An N-dimensional hypervolume of environmental conditions within which the organism can maintain a population”. There are two categories of environmental conditions: 1) physical/chemical (e.g., temperature, salinity, flow, pressure) and resources (e.g., nutrients, energy sources, space). The adaptive landscape for any one species includes: 1) the range of conditions in which the species functions competitively and can maintain a population, 2) an adaptive range in which the population can function but is no longer competitive, and 3) an adaptive limit at which individuals can not persist. For example, organisms transiently exposed to an adaptive limit of temperature elicit the heat shock stress response that serves for their recovery. However, they cannot persist at the elevated temperature. It is also evident that the adaptive limit for any one stressor may change when the organism is exposed simultaneously to multiple stressors.

Another essential requirement for using stress response as a monitoring tool is more complete understanding of the contribution of biotic interactions to physiological state and stress response. Only in the laboratory do microorganisms function in isolation. An example of biotic interaction changing the spectrum of stress response is a metabolic interaction commonly observed in anaerobic communities involving interspecies transfer of hydrogen or formate. In the absence of sulfate, sulfate-reducing bacteria can remain a dominant population by using a hydrogen-consuming microorganism (such as a methanogen) as an alternative electron acceptor [9,10]. The methanogen serves to pull the reaction by removal of an end-product of substrate oxidation (hydrogen or formate). Since many sulfate-reducing bacteria have the capacity to use hydrogen when respiring sulfate, these two alternative growth states alter the affect that increased hydrogen concentration has on cells when growing via sulfate respiration or syntrophically. In the first instance, hydrogen is a valuable substrate for growth. In the second, increased hydrogen inhibits growth. We anticipate that as stress response circuits are examined in environmentally relevant contexts, that much of our current understanding of stress response will be modified. Since many key environmental transformations, including the biodegradation of chlorinated hydrocarbon pollutants are sustained by syntrophic interactions [11], it is essential that the biological context of stress response be better constrained.

Our ability to “map” stress response in relationship to adaptive landscapes should also inform the concept of microbial species, address environmental factors that determine microbial biogeography, pathogen survival in the environment, and serve to monitor any system – whether natural or engineered – for the physiological status of the resident microorganisms. Thus, our intention in this short review is not to cover the many well studied stress response mechanisms, but to identify a few areas of investigative overlap that should have value in biotechnological applications in which monitoring stress response could contribute to process control.

What are signature responses to specific stressors? There are two general levels of possible interrogation of stress: 1) the immediate regulatory and physiological response and 2) subsequent changes in the transcriptome, proteome, metabolome, and cellular architecture. Immediate changes in protein and mRNA structure are part of the first line of defense (e.g., [12-

14]) but may not be easily monitored using available analytical methods. Alternative sigma factors also play a critical role in early adaptive response to transient and longer-term changes in environmental conditions. Many of these factors are conserved across wide phylogenetic groups and would offer general targets for assessing stress response. However, the identity of the alternative sigma factor regulons is needed in order to provide transcriptional and translational metrics of different stress response systems. To this end, the regulons for some better studied organisms are now being characterized using a combination of similarity searches for conserved promoter sequences, characterization of changes in protein composition (e.g., 2-D protein gel electrophoresis), microarray analysis, and more selective measurements of changes in transcription (e.g., reporter fusions)(see [15-17]). The paper by Rhodius et al. offers a nice perspective on the relationship between the lifestyle of the microorganisms and the complexity of the adaptive responsive mechanisms. For example, obligately intracellular *Mycoplasma* species contain only one housekeeping sigma factor and no alternative factors, whereas common soil-dwelling organisms such as streptomycetes exposed to constantly changing environmental conditions may have more than 60 alternative sigma factors. Their analysis of the σ^E regulon across nine Gram-negative genomes revealed a core response associated with the maintenance of outer membrane integrity, encoding for the synthesis and assembly of LPS and outer membrane proteins. However, the extended regulon for each organism included genes that appear to be niche specific, for example encoding pathogenic specific functions.

Research groups, including investigators supported by specific programs such as the Department of Energy's Genomics: GTL program are now developing the necessary bioinformatics tools to identify regulons controlled by specific sigma factors (<http://vimss.lbl.gov>). The heat shock stress response, having relevance to food processing and sterilization, is one of the better-characterized systems. Our most recent studies show that heat shock stress analysis can be applied to sulfate reducers found at metal contaminated sites to suggest general stress response pathways that could be relevant to other environmental stressors [18]. Other possible signatures of stress response include increased levels of expression of genes of lysogenic phage and transposons. For example, the global regulator H-NS has been reported to act directly on the transpososome to promote Tn10 transposition [19]. Continued advances in these areas should greatly enable the practical application of stress response data to systems level analysis of complex environmental

systems, identifying both core regulons likely having utility for general monitoring, and the extended niche specific regulon that in part defines individual species.

Environmental Systems. Biostimulation by addition of nutrient amendments to contaminated environments have recently started to focus on specific stressors that may affect biodegradation/biotransformation processes. Holmes et al. [20] monitored *nifD* gene for nitrogen fixation during acetate stimulation of organic- and nitrogen-poor subsurface sediments. While *nifD* expression decreased 100 fold after addition of ammonium it had no effect on rates of toluene degradation or Fe(III) reduction. Thermodynamic analysis of Cr(VI) exposure to sulfate reducers has also been shown to induce an inhibition of growth and energy production that is similar to oxidative stress responses [21]. This suggests that commonality in stress responses might provide strategies that can be used to maximize biodegradation/biotransformation processes in situ against specific contaminants without increasing biomass of the target organism. Bioaugmentation for biodegradation of carbon tetrachloride has also been shown to benefit from not only nutrient balance but also pH adjustments to avoid pH stress [22]. By adding a combination of alkali, acetate, phosphorus, and CT-degrader in a biocurtain strategy, carbon tetrachloride biodegradation in the groundwater passing through the biocurtain could be sustained at 100%.

Industrial Systems: Waste Treatment and Food Processing. Trickle-bed bioreactor systems for treating industrial wastewater typically have problems with media clogging from excessive biomass that greatly reduce the overall efficiency of the system. Recent studies have demonstrated salt stress inhibited bacterial growth but not substrate degradation by benzene-toluene-xylene degraders, suggesting that limited stress can be used to control bioreactor efficiency [23]. While short-term microbial adaptation to environmental stressors are protective at the cellular level, it may be disruptive in engineered bioreactors such as used for wastewater treatment [24]. For example, *cis-trans* isomerization of cell membranes can decrease rates of active transport due to decrease in membrane fluidity, altering linkage between cells and exopolymers (flocculation), and altering transport of hydrolytic enzymes out of cell, thereby altering biodegradation of extracellular material.

We also anticipate that there will be significant synergistic interaction in different areas of applied biotechnology that have a common interest in understanding microbial stress response. An area of great potential overlap is in food processing [25]. As food-processing technology increasingly emphasizes the use of less destructive food preservation methods, the production and storage of minimally processed foods increases the likelihood of microbial contamination. In response, there is a significant research literature addressing the stress response of important food born pathogens such as *Listeria monocytogenes* [13]. Its survival and growth at high osmolarity and at the low temperatures used for storage have received particular attention, examining different physiological roles of the alternative sigma factor, σ^B , in this and other Gram-positive bacteria. This sigma factor is involved in the resistance to a variety of environmental stresses (including heat, high osmolarity, high ethanol concentrations, high and low pH, and oxidizing agents) and has been recently reviewed by van Schaik and Abee [26], with an eye to possible using this knowledge to develop new food processing technology, possibly involving sequential preservation steps that do not activate stress response systems. Also, existing and emerging methods to monitor adverse physiological effects (stress) during large-scale production of recombinant proteins may have more general application. For example, a surface plasmon resonance biosensor for monitoring profiles of the heat-shock protein DnaK was shown to provide a measure of stress response associated with protein overproduction [27]. It is apparent that this kind of knowledge and associated technologies will have broad application in monitoring the physiological status of microbial populations, either to promote the growth of those that are favored or limiting the growth of those that are unwanted.

Conclusions

The identification of general and species-specific stress response regulatory elements and regulons should serve to identify appropriate metrics for process monitoring. We anticipate that the rapidly developing bioinformatics tools will continue to make this an achievable objective in the near term. However, the remaining challenge is to develop appropriate analytical methods to selectively measure response. Here we anticipate that new advances in areas such as proteomics (e.g., using high resolution mass spectroscopy) will be needed to effectively evaluate stress

response in open environmental systems. Amplification techniques suited to expression analysis may also have utility. RNA-based tools are also being used to infer metabolic rates from both pure cultures and environmental samples, e.g. carbon dioxide fixation rates in marine systems using *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase [or RUBISCO] gene) mRNA levels [28]. Once specific markers are identified, via these more labor intensive, costly, and broader techniques then we can use tools such as qPCR, which is more applicable for routine, cost-effective use. In the near term, it is more likely that application of stress response for process control will initially find application in engineered systems, such as bioreactors designed for waste treatment. An early exploratory example of using stress response as a monitoring tool is the immunochemical detection of GroEL (a highly conserved chaperone) in activated sludge reactors [24,29].

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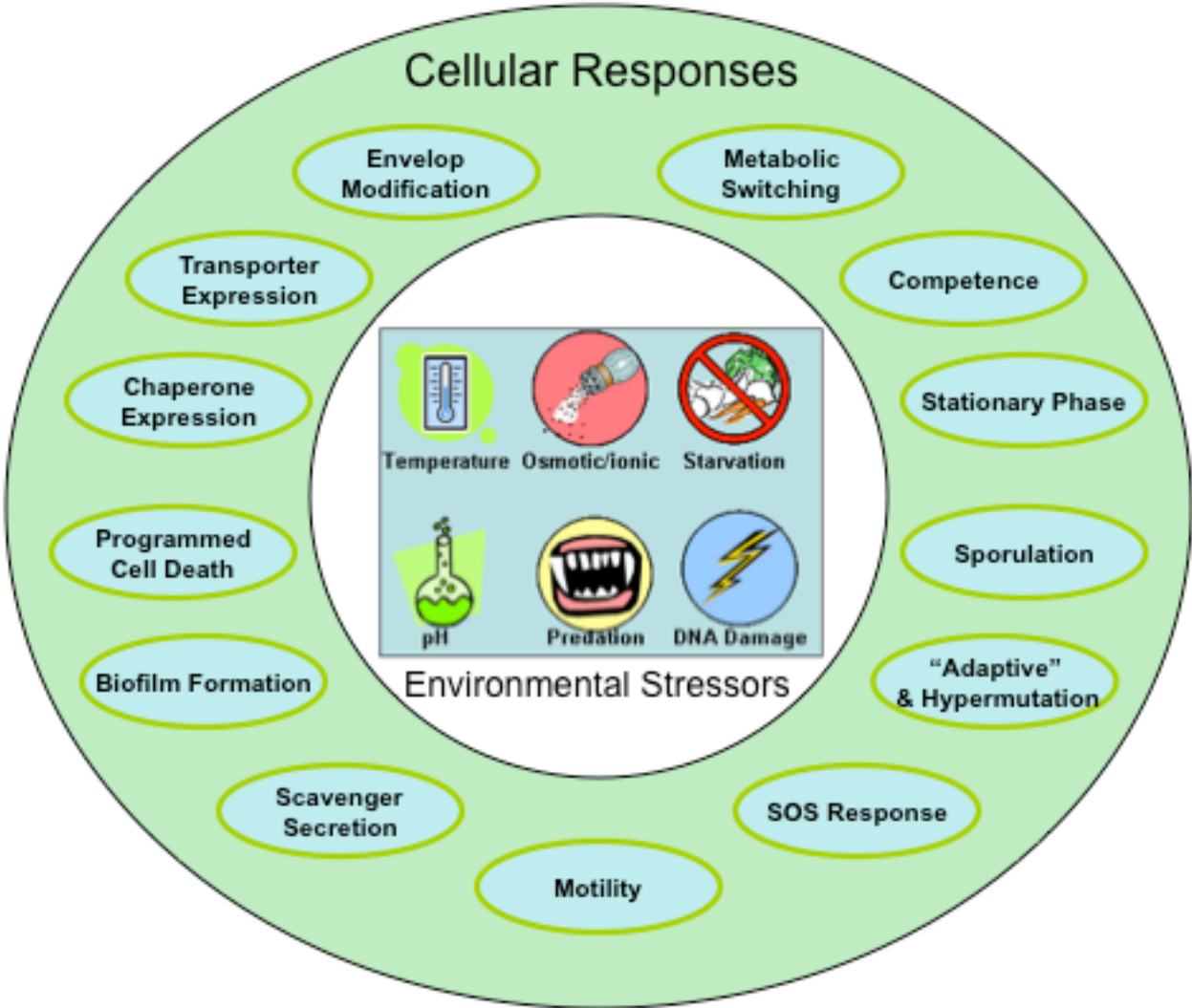
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Figure 1. Multiple response pathways to environmental stress



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