Perspectives and vision for strain selection in bioaugmentation

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Notwithstanding the phenomenally large and ever-increasing resource of pollutant-degrading microbial isolates in laboratories around the globe, inoculum survival remains the ‘Achilles’ heel’ for bioaugmentation of contaminated land. Considerable effort has been invested into inoculum strain selection to facilitate pollutant biodegradation, ranging from the isolation of ‘superbugs,’ which are microorganisms highly resilient to environmental stresses, harboring catabolically superior pollutant-degrading enzymes, to the other extreme in ‘priming,’ where pollutant degradation is carried out through the addition of soil enriched with an undefined consortium of pollutant-degrading microorganisms.

The past four years (2001–2004) have seen 172 scientific papers including the keyword ‘bioaugmentation’ (ISI Web of Science) – nearly double that of the previous four-year period. Nevertheless, this increase has yet to correlate with an increase in the implementation of field-scale bioaugmentation. In this article, we discuss our perspective on the pinch-point for bioaugmentation – strain selection. We suggest that many of the challenges of bioaugmentation can be attacked with greater efficacy through the manipulation of a select subset of the ‘superbug’ population. We propose that the future of bioaugmentation rests in the coordinated efforts of the international scientific community to harness the unrealized potential of the superbugs, rescuing them from a destiny of serial ‘proof of concept’ studies. We also highlight two strain-selection techniques that show great promise for further enhancing the efficacy and applicability of bioaugmentation in the field.

Bioaugmentation is an approach rooted in the ancient art of food preparation. Most notably, cheese, yoghurt and beer production employ small quantities of the previous batch of fermented product to initiate the process once again, thereby reproducing with high precision the same product with each successive batch. For millennia, the ‘starter culture’ for milk fermentation was supplied from the fortuitous storage of cow or goat milk in an animal skin bag. It was not until insights provided by Louis Pasteur in the later half of the 19th century, that dairy science was able to transform from the black-box art form mastered by few, to a six billion dollar (US), scientifically precise industry, mastered by many.

Despite our long cultural history of bioaugmentation, it is only recently that we have attempted scientifically to engineer many of these processes. Bioaugmentation of polluted soil is an approach whereby microorganisms are amended to a contaminated site to hasten the degradation of pollutants. It is through the introduction of excess, active, degrader microorganisms that the pollutant is mineralized, thereby providing remediation at a faster rate than would otherwise occur by means of the indigenous microorganisms. We direct the reader to van Veen *et al.*, for an excellent, comprehensive review of bioaugmentation techniques [1].

Strain selection for bioaugmentation is contingent on our understanding of not only microbial ecology but society. Clearly, recent developments in molecular microbial ecology have greatly assisted our ability to track strains, interrogate environmental systems and appreciate the complexity of soil microbiology in ways that only five years ago were impossible. If a plentiful supply of superbugs are available for exploitation, and our understanding of the target system is increasing rapidly, why are there so few examples of field-scale bioaugmentation? In this paper, we propose that much progress can be gained through a coordinated exploitation of the current knowledge base, molecular biology techniques and global culture collections.

Strain selection

Strain selection is founded on the principle that certain microorganisms are better suited for particular (catabolic) tasks and environments than others. Traditional bioaugmentation has achieved its greatest results through repeated application of highly competent pollutant-degrading bacteria [2,3]. Arguably, the high frequency of bioaugmentation events is necessary owing to poor survival and activity of the inoculum, which, among a myriad of other reasons, is ultimately founded on strain selection. When designing a bioaugmentation regimen, for obvious reasons, strain selection is confined to those species that are not ‘linked’ to human pathogens (i.e. share the same genus and species name); however, it is often the case that a ‘superbug’ is closely related to a human pathogen, precluding its field implementation [4]. This is exemplified by *Pseudomonas aeruginosa*, a versatile Gram-negative bacterium that grows in soil, marshes and coastal marine habitats and has been utilized as a plant growth-promoting rhizobacterium. Specifically, *Pseudomonas aeruginosa* strain IE-6 was

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shown to suppress the root-infecting fungi *Macrophomina phaseolina*, *Fusarium oxysporum*, *Fusarium solani* and *Rhizoctonia solani* in tomato plants [5]. However, a range of different strains of *P. aeruginosa* are also known to infect the lungs of cystic fibrosis sufferers, where it causes inflammation and episodes of intense breathing problems which can be potentially fatal [6]. Hence, strain selection must not be based on performance alone.

Heirloom strains

The search for novel microorganisms with exceptional catabolic or survival abilities is an ongoing pursuit of many laboratories worldwide. This effort has generated many hundreds of thousands of 'ordinary' microorganisms, along with a notable few with exceptional abilities (i.e. superbugs). A subset of the superbug population, denoted as 'Heirloom' microorganisms, are superbugs that are maintained and handed down from generation to generation within a research group – with each successive transfer adding phenotypic and genotypic details and genetic modifications to its meta-dossier. These Heirloom superbugs are particularly attractive for bioaugmentation for several reasons: they are usually easily cultured, fast growing, thoroughly characterized and accessible worldwide through one of the many culture collections (e.g. American Type Culture Collection (ATCC), Belgian Co-ordinated Collections of Microorganisms (BCCM), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and Czech Collection of Microorganisms (CCM)).

Arguably, one of the most exemplary Heirloom microorganisms is *Deinococcus radiodurans*. This bacterium has been under increasing scientific scrutiny for nearly five decades, accruing a thick phenotypic [7] and genotypic dossier [8]. The bacterium touts a dizzying array of talents, ranging from its natural resistance to genotoxic chemicals, oxidative damage, high levels of ionizing and ultraviolet radiation and dehydration to its genetically engineered acquisitions of tolerance to high temperatures, ability to biodegrade toluene and detoxify a range of potentially hazardous metals [e.g. mercury (II), iron (III), uranium (VI) and chromium (VI)] [9,10]. Ultimately, *D. radiodurans* is being groomed for one of the most difficult of pollutant challenges – bioaugmentation into high-temperature radioactive mixed wastes (i.e. organic and metal pollutants). Despite its awesome potential to remediate among the most challenging and hazardous polluted sites, it is not likely to be readily adopted by problem holders worldwide because of its genetic modifications and unknown impact on the environment. Hence, this superbug could very well find itself in Heirloom purgatory, where it might remain indefinitely, forever stymieing the interests of researchers, funding bodies and problem holders [11,12].

One solution to this problem, in principle supported by Barac *et al.* [13] and Blumenroth and Wagner-Dobler [14], is that the scientific community coordinates its research efforts on Heirloom microorganisms. The pool of Heirloom species would be chosen based on their amenability to manipulation, exceptional catabolic ability, current knowledge base, tolerance to environmental stresses,

technical ease of use and lack of public health threat (e.g. *Pseudomonas* sp. strain ADP, *Pseudomonas* sp. NCIB 9816–4, *Burkholderia xenovorans* LB400, *Phanerochaete chrysosporium* strain RP78, *Methylococcus capsulatus* (Bath), *Geobacter sulfurreducens*, *Deinococcus radiodurans*). The Heirloom collection would constitute a dynamic pool of superbugs, thoroughly tested for their suitability for field use as well as optimized for enhanced survival and activity in the environment through (genetic) manipulation. The proposal would establish the microbial equivalent of open-source coding (e.g. LINUX), focusing the international research effort into providing a toolbox of workhorse microorganisms upon which all field releases could be carried out, affording the greatest likelihood of achieving the desired objectives while providing the best opportunity to address the safety concerns of the public. In the end, the approach would impart greater value for money to the research-funding body, provide the problem holder with a scientifically sound, cost-effective technology to remediate polluted sites and ultimately inspire creative and innovative science, in large part owing to *a priori* knowledge that the finished product stands a reasonable chance of contributing to the remediation of contaminated land.

In rhizo-directed strain selection

In a majority of cases, superbugs are isolated through a standard procedure of batch-enrichment culture, which relies on the ability of the isolate to grow rapidly on a specific carbon source in defined chemical and environmental laboratory conditions. Although highly effective in generating superbugs, the approach operates in a vacuum from the natural environment. In an effort to advance the techniques of strain selection beyond traditional batch enrichment, researchers have turned to a relatively new approach, which incorporates the benefit(s) of plants as witnessed in phytoremediation with optimized biodegradative strains [15], called *in rhizo*-directed strain selection. The approach utilizes a microorganism with the capacity to grow on one or more root exudates within the rhizosphere in addition to degrading the relevant pollutant. The microorganisms, once inoculated into the appropriate plant rhizosphere, would express the pollutant-degrading enzymes, thereby decontaminating the soil while growing on the plant root exudates to sustain its population and metabolic activity. An added benefit of including plants in the bioaugmentation approach is the supply of additional secondary metabolites and root exudates, which might also fortuitously stimulate and maintain the induction of pollutant-degrading enzymes *in situ* [16,17]. The inoculum, in return, can provide protection to the plant against phytotoxic chemicals and plant pathogens. The synergistic relationship between the plant and the inoculum established by this approach is arguably more resilient, sustainable and cost-effective than the standard bioaugmentation approach. Reliance of the inoculum on plant exudates for survival precludes its unintended dispersal to nontarget soils, thereby providing a mechanism for addressing public safety concerns.

Kuiper *et al.* [18] were the first to methodically select a strain indigenous to a host plant's rhizosphere that

exhibited preferential growth on the plant root exudates, good survival in the rhizosphere and enhanced pollutant-degrading ability. To a similar end, Narasimhan *et al.* [19] engineered a bacterium to utilize the predominant root exudates of *Arabidopsis* for the remediation of polychlorinated biphenyl (PCB) in soil. This nutritional bias for the inoculum provides a selective advantage for the strain while in the *Arabidopsis* rhizosphere. The inoculum, a PCB-degrading *Pseudomonas putida* PML2, was then capable of degrading PCBs upon augmentation into the *Arabidopsis* rhizosphere, while growing on phenylpropenoids, the main root exudates, as a 'preferential' growth substrate [14]. This study highlights one of the most promising approaches for the future of bioaugmentation: the combination of a sustainable, semiselective nutrient source in plant exudates with the rhizosphere competence of a pollutant-degrading 'superbug' [17].

Additional techniques can be employed *in situ* to achieve superior strains for bioaugmentation. de Weert *et al.* [20] demonstrated a novel method of enhancing competitive root-tip colonization in a *Pseudomonas fluorescens* strain through repeated cycling of the strain and mutant derivatives on the plant root *in situ*. The authors observed a 100- to 1000-fold increase in the strain's root-tip-colonizing ability in tomato and grass plants. The authors concluded that enhanced competitive root-tip colonization was a result of the accumulation of nonlethal mutations, which enabled the strain to rapidly adapt to the plant rhizosphere. This approach to strain selection is particularly attractive because it is rapid, inexpensive, does not require genetic engineering and is readily applicable to Heirloom strains.

Non-traditional strain selection

Up to this point, we have provided our vision for the future of bioaugmentation: Heirloom species and *in rhizo*-directed strain selection. However, we recognize that significant potential rests in a less traditional approach, termed 'priming'. Priming is generally described as predisposing an isolate or population of microorganisms to future conditions in which they are designed to perform a function. Priming is routinely used in probiotic yogurt culture preparation, where *Lactococcus* or *Bifidobacterium* cultures are subjected to the mildly acidic environment of the yogurt, 'priming' the inoculum for the harsh, acidic conditions within the stomach [21,22]. This pretreatment, albeit unnecessary for acid-tolerant strains, is essential for the viable transit of nonacid-tolerant strains through the stomach into the intestines [21].

The priming approach has also been effectively demonstrated in environmental systems, whereby a portion of clean soil is enriched for pollutant-degrading microorganisms by repeated biostimulation with the relevant pollutant(s). The resulting soil itself, with its highly competent degrader microbial community, is then used as the inoculum for the target polluted soil [23]. This approach has several advantages: (i) the inoculum is a consortium of indigenous microorganisms which is potentially more resilient to stress than a single isolate; (ii) the 'primed' consortium is maintained within its native soil, potentially enhancing its survival in the target soil; and

(iii) inclusion of unculturable microorganisms, which might contain one or more highly competent pollutant degraders. In addition, the approach is theoretically expandable to solving the issue of co-contaminated soil (i.e. one with more than one pollutant), which might necessitate the combination of singly primed soils or one soil primed to degrade multiple contaminants.

Takeuchi *et al.* [24] were among the first to apply priming to contaminated groundwater. The authors initially demonstrated cometabolism of trichloroethylene (TCE) by methanotrophs from a borehole in a contaminated aquifer. They subsequently amended a poorly performing borehole with groundwater from the TCE-degrading borehole, resulting in a rapid decline in the TCE groundwater concentration. Treatment success was attributed to the activity of the bioaugmented indigenous methanotrophs within the 'primed' TCE-degrading groundwater. Albeit a return to the 'black box' method of remediation, instinctively avoided by scientists, this approach is attractive from a practical perspective; it is technically accessible, easily implemented, inexpensive and environmentally friendly. It remains to be seen how effective different matrices respond to priming (e.g. clay-primed soil augmented into sandy target soil, or high pH primed soil augmented into neutral or low pH target soil). Moreover, the approach has not been suitably tested for potential scale-up to address large contaminated sites; however, priming clearly warrants a more thorough investigation to answer these and other practical questions.

Conclusion

In time, it is hoped that bioaugmentation can fulfill its potential to remediate contaminated soils and water, *in situ*. Many of the limitations to its applicability in the field are the result of an uncoordinated effort to address the social concerns of bioremediation along with the scientific interests. To this end, we propose that exploitation of Heirloom species should be coordinated between research laboratories, providing both scientists and regulators with a predictable, (globally) tested selection of microorganisms, which would facilitate pollutant remediation as well as its public acceptance. In addition, we highlight two non-traditional approaches to strain selection, termed *in rhizo*-directed strain selection and priming, which demonstrate great promise for remediation of polluted matrices but remain largely on the periphery of the discipline.

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