Phylogenetic and Functional Fluorescent Probes for Culture-Independent Identification and Cell Sorting of Indigenous 1,4-Dioxane Degraders

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Background/Objectives. Recent studies with molecular tools and isotopic analysis have unveiled the occurrences of indigenous 1,4-dioxane (dioxane) degrading microorganisms might be more widespread than previously assumed at the contaminated field. Isolating and characterizing bacteria capable of degrading dioxane is helpful to understand their metabolic and physiological idiosyncrasies and advance the development on novel bioremediation techniques. However, this is not an easy task if one relies on traditional isolation techniques (e.g., enrichment and serial dilution for separating colonies on plates) due to the fastidious nature of many indigenous organisms. In general, less than 5% of known bacteria can grow on plates. In this study, we aimed to develop sensitive and specific fluorescent probes to identify and sort indigenous dioxane degraders in enriched environmental samples using advanced flow cytometry and cell sorting technique.

Approach/Activities. In parallel, two sets of probes were designed: (i) phylogenetic oligonucleotide probes targeting 16S rRNA genes, and (ii) functional polynucleotide probes targeting the *thmA/dxmA* mRNA transcripts encoding the large hydroxylase of dioxane or tetrahydrofuran monooxygenases. In situ hybridization was optimized by numerically computing and empirically assessing the annealing and dissociation temperatures of the probe-target hybrids, as well as the concentrations of formamide in the hybridization buffer. Coverage and specificity of the probe sets were evaluated by epifluorescence microscopy using pure bacterial strains harboring the targeted genes or not and reference strains (e.g., *Psedonocardia dioxanivorans* CB1190) that were induced and uninduced by dioxane. Within 4 hours after in situ hybridization, resuspended cells were diluted with sterile PBS buffer to a range of 10^3 to 10^4 cells/µL, and submitted for flow cytometry analysis. Single cells targeted by our developed fluorescent probes (either polynucleotide mRNA probes or oligonucleotide rRNA probes) were sorted into a microtiter plate. Unstained negative controls were employed to discern background fluorescence emission.

Results/Lessons Learned. With our *thmA/dxmA* probes, we were able to label known dioxane-degrading strains (e.g., *Psedonocardia dioxanivorans* CB1190), and also separate putative degraders from the enriched consortium prepared with aquifer samples from contaminated sites. Next generation sequencing technology was applied to analyze the 16S rRNA genes of these sorted bacteria. Known degraders, such as *Flavobacterium* spp., *Mycobacterium* spp., *Afipia* spp. and *Pseudonocardia* spp. were widely observed in these sorted cells originated from the contaminated sites. Meanwhile, we developed probes to target specific known degrading microorganisms (e.g., *Pseudonocardia* spp. and *Mycobacterium* spp.), and the high sensitivity and great specificity of the probes were verified with fluorescence microscopy and flow cytometry. These probes suggest great potential to rapidly detect 1,4-dioxane degraders, economically identify them, and stably predict bioremediation. This study advanced our understanding of the phylogenetic and metabolic diversity of indigenous dioxane degraders and availed us with innovative molecular technique for *in situ* enumeration and detection.