Kill•Spill

Integrated solutions for combating marine oil spills

Deliverable D2.1

First validation report of the Kill•Spill Chip

This project is supported by the European Union under the Food, Agriculture and Fisheries and Biotechnology theme of the 7th Framework Programme for Research and Technological Development under GA no. 312139

Work package	WP2 Development of biosensors and in-situ monitoring tools to determine biodegradation efficiency
Deliverable no.	D2.1
Deliverable title	First validation report of the Kill•Spill Chip
Due date:	2014-06-30 (Month 18)
Actual submission date:	2014-10-23
Start date of project:	2013-01-01
Deliverable Lead Beneficiary (Organisation name)	Bangor University
Participant(s) (Partner short names)	Bangor
Author(s) in alphabetic order:	Prof P Golyshin, Tatyana Chernikova
Contact for queries:	Prof P Golyshin School of Biological Sciences, Bangor University ECW Bldg, 2nd floor, Deiniol Rd., Bangor LL57 2UW Gwynedd, UK T: +44 (0) 1248 38 3629 E: p.golyshin@bango.ac.uk
Dissemination Level: (PUblic, Restricted to other Programmes Participants, REstricted to a group specified by the consortium, COnfidential only for members of the consortium)	PU
Deliverable Status:	Final 2014-10-23

Table of Content

List of Figures

1 About this deliverable

The purpose of this work is to develop and validate microarray chip that will be used to detect genes coding for enzymes for particular reactions in oil biodegradation pathways in microbial communities in the environmental samples and experimental setups made up in the Kill•Spill project. This deliverable provides the summary of experimental validation of Kill•Spill chip microarray. In the following sections, we describe principal steps involved of this procedure.

2 Description

2.1 Introduction

Contamination of marine environments with petroleum oil is of particular concern because of its global scale and the severity of ecological consequences it may cause. Microorganisms play a major role in the process of oil biodegradation in the contaminated sites, and it is therefore important to assess the biodegradation capacity of microorganisms readily available in pristine and polluted systems to rationally design the interventions such as bioaugmentation or biostimulation. One of the methods to achieve that is the use of microarray-based genomic technologies that represent a powerful tool for analysis of metabolic potential of microbial community in environmental samples (Zhou, 2008). The genome sequences of several important marine hydrocarbon degraders (and their communities) have been determined in recent years, steadily increasing the numbers of genes to be targeted and putting them into the context of metabolic biodegradation networks (Brooijmans et al., 2009). The information derived from those studies along with the information from databases on key catabolic genes on aliphatic and aromatic degradation was used to develop/upgrade the microarray to provide an insight into the (meta)genomic landscape of marine oil-degrading microbial communities.

2.2 Experimental procedures

The activities in frames of Deliverable 2.1 involved the production and validation of Kill•Spill microarray.

2.2.1 Probe design

For probe design we used the set of 1600 genes for aromatic catabolic gene families and for alkane degradation pathways that have been experimentally validated earlier (Vilchez-Vargas et al., 2013). This gene set has been amended with the additional set of 694 genes revealed from recent sequencing of positive fosmid clones from PAH-exposed soils and sediments and newly characterised proteins from public databases. The total set of genes has also been amended with 81 gene probes derived from recently sequenced genomes of several hydrocarbon degrading strains (*Alcanivorax borkumensis* SK2, *Thalassolituus oleivorans* Mil1, *Oleispira Antarctica* RB8, *Marinobacter hydrocarbonoclasticus* VT8 and *Cycloclasticus zancles* ME-7) to make 2375 probes in total. The gene probes (50-mer oligonucleotides) for the new gene candidates' dataset have been designed according to Vilchez-Vargas (Vilchez-Vargas et al., 2013). The main protein families/superfamilies to be detected with the Kill•Spill chip were: Rieske non-haem iron ring hydroxylating (di)oxygenases of the vicinal chelate superfamily (RHDO), extradiol dioxygenases of the vicinal chelate superfamily (EXDOI), intradiol dioxygenases (INDO), soluble di-iron aromatic ring hydroxylating monooxygenases, ferredoxins of multicomponent aromatic degradation enzymes (FERRE), muconate cyclo-isomerases (MCIS), maleylacetate reductases (MARC), alkane hydroxylases of the integral membrane-bound

monooxygenases (ALKB). Importantly, the core of the array is represented by probes to detect genes for proteins whose activities have been experimentally validated in the lab.

2.2.2 Probe preparation and printing

Probes (50 mer 5'-amine-modified oligonucleotides) were synthesized by MacroSynth GmbH (Switzerland). Probes were printed on CodeLink activated slides (SurModics, Eden Prairie, USA) using MicroGrid TAS II spotter (BioRobotics, Germany) at the University of Frankfurt (Frankfurt, Germany). Coupling of DNA probes was performed by overnight incubation of slides in saturated NaCl chamber. Post-coupling processing included the blocking of residual reactive groups and was done as follows: slides were washed with 4x SSC (Saline-Sodium Citrate buffer), 0.1% SDS (Sodium Dodecyl Sulfate) for 30 min, then rinsed briefly with deionized water and dried by centrifugation for 3 min at low-speed centrifuge.

Array technical details. The gridding tables (Excel files) with gene and corresponding protein sequences as well as .GAL file for referencing the arrayed probes can be acquired from Kill•Spill Partner *Bangor University* upon request.

2.2.3 Strains used for validation

Five hydrocarbon degrading bacteria with available genomes were used for validation of Kill•Spill microarray and included *Alcanivorax borkumensis* SK2, *Thalassolituus oleivorans* Mil1, *Oleispira antarctica* RB8, *Marinobacter hydrocarbonoclasticus* VT8 and *Cycloclasticus zancles* ME-7. Strains were grown in ONR7a medium supplemented with either aliphatic hydrocarbons (0.2% of hexadecane in case of *A. borkumensis* SK2, *T. oleivorans* Mil1, *M. hydrocarbonoclasticus* VT8 and 0.2% tetradecane in case of *O. antarctica* RB8,) or aromatic hydrocarbons (0.1% 2-methylnaphtalene in case of *C. zancles* ME-7) according to growth conditions for each strain. Cells were harvested during late exponential growth phase. Total DNA was extracted from each of five strains using Meta-G-Nome DNA isolation kit (Epicentre). The quality and quantity of the DNA samples were analysed on 0.8% gel and using Quanti-iT dsDNA Assay kit (Invitrogen) with fluorescence spectrophotometer Cary Eclipse (Varian).

2.2.4 Samples preparation

Whole genome amplification was carried out using the REPLI-g Ultrafast kit (Qiagen) according to manufacturer's instruction. Following amplification, DNA samples were purified by phenol/chloroform extraction. After mixing samples with equal volume of phenol/chloroform solution, the mixture was centrifuged and aqueous phase was transferred to a new tube. Finally, DNA was precipitated by adding three volumes of ethanol and 1:10 volume of 3 M sodium acetate. The DNA pellet was washed with 80 % ethanol and re-suspended in 100 µl of sterile water. The resulting genomic DNA was heat-fragmented at 95° C for up to 1.5 h. The aliquot of each digestion reactions was analysed on agarose gel and digestion reactions were stopped if the majority of DNA fragments had a size range of 200-1000 bp. The resulted DNA was precipitated, suspended in 45 µl of MilliQ water and labelled by direct incorporation of Cy5-conjugated dUTP (GE Healthcare) using terminal deoxynucleotidyl transferase (Thermo Scientific, UK). The labelled DNA fragments were precipitated and re-suspended in 20 µl of MilliQ water.

2.2.5 Hybridization and washing

Prior to hybridization, labelled DNA was incubated with herring sperm DNA (Invitrogen) for 5 min at 95° C and then 80 µl of hybridization buffer was added. For hybridization, slides were inserted into hybridization chamber and covered by coverslips. Labelled samples were added by diffusion.

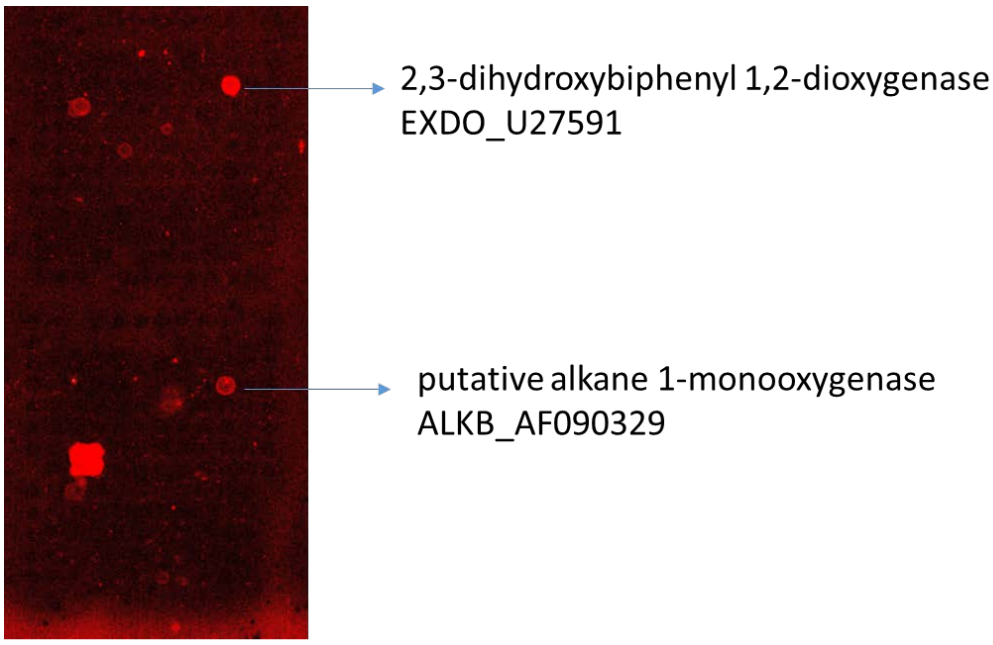
Hybridization was performed at 55° C for 18 h using hybridization buffer consisting of 15% (v/v) dimethylsulfoxide, 25% (v/v) formamide, 1.25 x SSC (190 mM sodium chloride plus 20 mM tri-sodium citrate equivalent to sodium concentration of 250 mM), 0.15% SDS, 0.15% Tween20, 880 mM betaine, 5x TE buffer (50 mM Tris-HCL, 5 mM EDTA) and 0.1 mg/mL BSA in aqueous solution. Following hybridization, slides were washed 5 min at 42 C in 1x SSC 0.3% SDS, twice in 1x SSC (1 min, 42 C), in 0.5x SSC (1 min, 20° C), in 0.1x SSC 0.3% SDS (1 min, 42° C) and finally twice in 0.1x SSC (1 min, 20° C). Slides were dried at low speed in centrifuge for 30 seconds.

2.2.6 Scanning and analysis

The microarrays were scanned using a High Resolution Microarray Scanner (Agilent Technologies, CA, USA). The scanner output images were analysed using ImaGene software v5.2.2 (Biodiscovery, Los Angeles, CA). A grid of individual circles defining the location of each spot on the array was superimposed on the image to quantify the intensity of each single spot. The mean signal intensity and the background intensity were determined for each spot.

2.3 Results

The Kill•Spill chip microarray was designed and produced. This microarray comprises 2375 probes, which were printed across the glass surface. To validate the Kill•Spill chip microarray, we hybridized this array with labelled DNA mixture derived from five hydrocarbon degrading strains with available genomes. All steps of microarray experiment were optimized and standardized. We have obtained good results with CodeLink slides. Figure 1 shows some results of hybridization in which Cy5 was used to label aliquots of DNA from strains used for validation. Typically, the hybridization signals indicated as examples of “positive” hits were detected in all three technical replicates. In frame of the project, this microarray protocol will be used to analyse different experimental samples established in further work packages to give an overview of catabolic gene subfamilies involved in oil degradation at the environmental/experimental polluted sites.



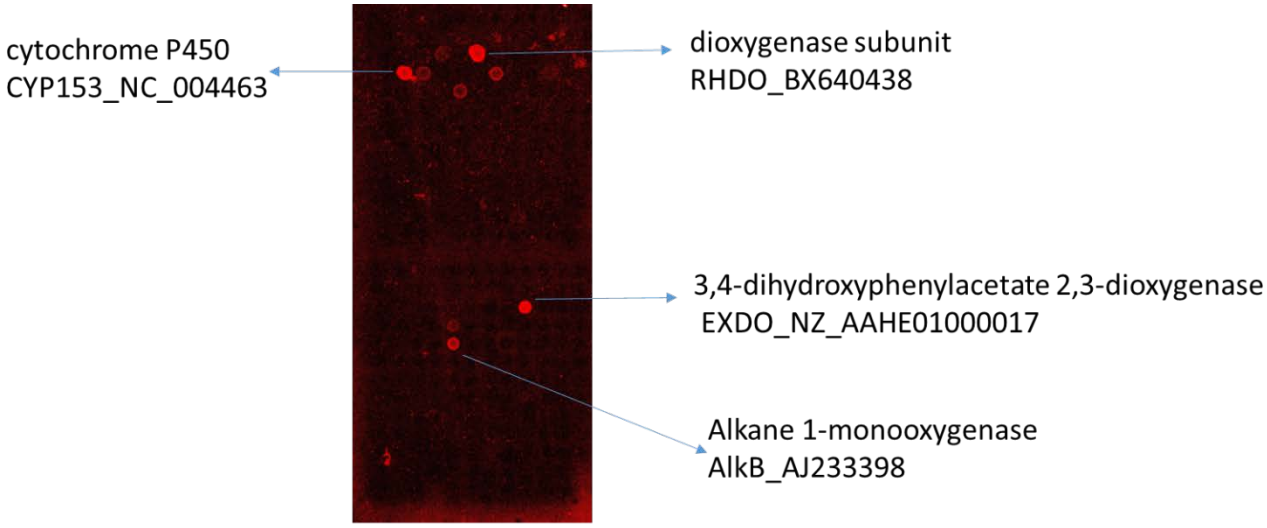


Figure 1 **Selected subsections of a representative microarray slide.**
The arrays were hybridized with Cy5-labeled target DNA from type strains selected for validation. Only spots present in all three technical replicates were considered positive.

2.4 Conclusion

The first validation of the Kill•Spill Chip has been performed. We were able to detect, as expected, a number of signals that correspond to the genes homologous to those in the genomes of tested type strains.

The Chip will be used to accomplish the work with contaminated marine sediment and seawater samples subjected to the meso- and macrocosm decontamination trials. Furthermore, the data to be produced using the Kill•Spill Chip will be integrated with independent OMICS approaches (DNA sequencing, proteome and metabolite profiling).

3 References

Zhou J. Microarrays for bacterial detection and microbial community analysis. Current Opinion in Microbiology. 2008, 6:288-294.

Brooijmans RJ, Pastink MI, Siezen RJ. Hydrocarbon-degrading bacteria: the oil-spill clean-up crew. Microb Biotechnol. 2009 Nov: 2(6): 587-94.

Vilchez-Vargas R, Geffers R, Suárez-Diez M, Conte I, Waliczek A, Kaser VS, Kralova M, Junca H, Pieper DH. Analysis of the microbial gene landscape and transcriptome for aromatic pollutants and alkane degradation using a novel internally calibrated microarray system. Environ Microbiol. 2013 Apr;15(4):1016-39.