Kill•Spill

Integrated Biotechnological Solutions for Combating Marine Oil Spills

Deliverable D2.9
Improved variants of the TodS sensor adapted to recognize high molecular weight PAHs

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1 About this Deliverable

A main objective of WP2 is to develop tools to monitor the presence of hydrocarbons in environmental samples from different origins, especially sea-water and sediments. This deliverable is focused on the detection of aromatic compounds with 2, 3 and 4 rings, and is based on the efficiency of the *P. putida* DOT-T1E toluene-responsive system that uses the TodS/TodT regulators. This system is the core structure of already available tools to sense toluene at very low concentrations (see Deliverable 2.4). Herein, we test the possibility to obtain variants of the response regulator TodS able to recognize as effector aromatic compounds with two or more rings. It is worth noting that in the current stage of the Kill•Spill project, two efficient biosensors detecting naphthalene, phenanthrene and anthracene have already been developed and are available as monitoring tools, and new variants of these systems are in progress (Table 1). The results obtained with the latest versions of these biosensors are also presented in section 2.1 of this Deliverable. Altogether, this set of biosensors largely covers the needs for PAH monitoring tools and reduces the relevance of this specific Deliverable. We have however tested the possibility of obtaining PAH-responsive TodS variants (sections 2.2 and 2.3), as initially proposed in the DoW.

Table 1 Developed PAH-monitoring tools within the Kill-Spill project

Biosensor	Control module	Detected compounds (detection limit)	Detection in mixtures	Host bacteria
pKSN-1	*phnR-PphnS::gfp*	Naphthalene (31.6 mg/L) Methyl-naphthalene	Petrol (0.015 mg/L) [1] Diesel (0.29 mg/L) [1]	*Burkholderia* sp. MS3
pKSPA-1	*PpahA::gfp*	Salicylic acid (0.25 mg/L) Naphthalene (4,4 mg/L) Methyl-naphthalene Phenanthrene (1,6 mg/L)	Petrol (0.015 mg/L)[2] Diesel (0.04 mg/L)[2] Gela water Gela sediments North Sea oil	*Novosphingobium* sp. HR1a
pKSPA-R[3]	*PpahR-pahR; PpahR::gfp*	3-methylbenzoate *p*- and *m*-xylene Salicylic acid Naphthalene Methyl-naphthalene Biphenyl Phenanthrene	Petrol (0.009 mg/L)[2] Diesel (0.0035mg/L)[2] Gela water Gela sediments North Sea oil	*Novosphingobium* sp. HR1a

[1] Total naphthalene content
[2] Total PAH content
[3] Discussed in section 2.1

2 Results

2.1 pKSPA-R, a fast and sensitive biosensor for the detection of PAHs

Novosphingobium pKSPA-R is a new biosensor for mono- and polycyclic aromatic compounds. It originates from the gene cluster for the PAH degradation pathway in *Novosphingobium* sp. HR1a. In this cluster, the naphthalene degradation pathway is under the control of *pahR* regulatory gene. In the presence of a number of aromatic compounds, PahR activates P_{pahA} promoter of the naphthalene degradation operon, and also activates its own expression through the P_{pahR} promoter. This self-regulation is controlled by the presence of a number of PAHs in the medium, providing the possibility to use the PahR/P_{pahR} system as the basis for a new PAH-responsive biosensor. The regulatory part of this new biosensor is thus composed by the regulatory gene (*pahR*) and its target promoter P_{pahR} fused to the green fluorescence protein (Figure 1).

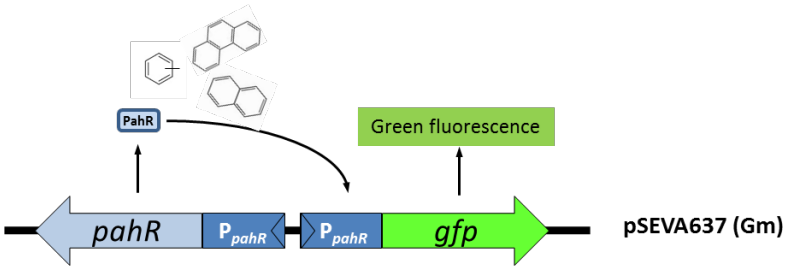

Figure 1 Scheme of the pKSA-R biosensor.
The PahR regulator in plasmid pKSA-R, which has been cloned from *Novosphingobium* sp. HR1a genome, responds to the presence of mono- and polycyclic aromatic compounds by activating transcription from the P_{pahR} promoter. Therefore, the P_{pahR} *-gfp* reporter fusion located in the same plasmid responds to these compounds by producing the GFP protein.

This biosensor is able to detect monocyclic (methyl-benzoate, *m- p- o*-xylenes and salicylate) and polycyclic aromatic compounds (e.g. naphthalene, phenanthrene and derivatives). The PahR regulator senses the presence of these compounds and responds by triggering the expression of the GFP protein from promoter P_{pahR} (Figure 2). Interestingly, high response levels were already obtained after 5 hours of incubation with the pollutants, whilst 24 hours were required to obtain a sound response with the previously developed pKSA-1 biosensor. As expected, the new pKSA-R biosensor is also able to detect PAHs present in complex mixtures; its sensitivity to detect hydrocarbons present in diesel, fuel or crude oil was compared with that of the previously developed pKSA-1 biosensor, which was described in Deliverable D2.4. The results, presented in Figure 3, show that this new sensor is more sensitive than pKSA-1 with these samples. We have calculated that its detection limit for total PAHs contained in petrol is 9 ppb, and 3.5 ppb for total PAHs contained in diesel. This biosensor could also be activated in the presence of water and sediments from the Gela spill (<5% v/v), and of water saturated with North Sea oil (<3% v/v). *Novosphingobium* sp. HR1a bearing the pKSA-R plasmid is so far the most sensitive biosensor obtained in this project for the detection of PAHs in hydrocarbon contaminated samples.

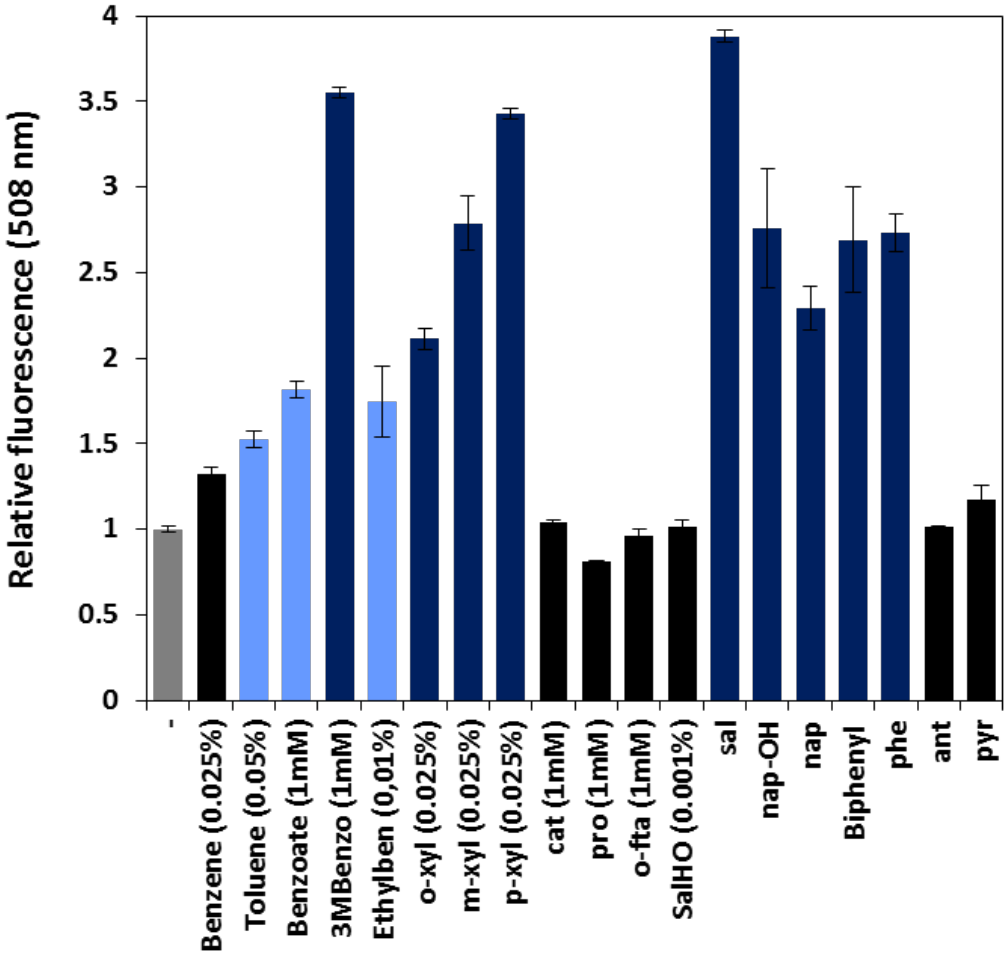

Figure 2 **Fluorescence emission of a bacterial strain containing the reporter plasmid pKSA-R in the absence or presence of different aromatic compounds.**
This plasmid includes the PahR/P_{pahR}::*gfp* reporter system that allows production of the Green Fluorescent Protein (GFP) in response to the presence of different aromatic compounds. Compounds were directly added to the growth medium at the indicated final concentration and fluorescence was determined after 5 hours incubation. Colour code: grey, non-amended control; black, compounds not detected by the sensor; light blue, compounds producing an intermediate response; dark blue, compounds producing a strong response. Cat, catechol; pro, protocatechuate; o-fta, *o*-phthalate; SalOH, salycialdehyde; sal, salycilate; nap-OH, hydroxynaphthalene; nap, naphthalene; phe, phenanthrene; ant, anthracene; pyr, pyrene.

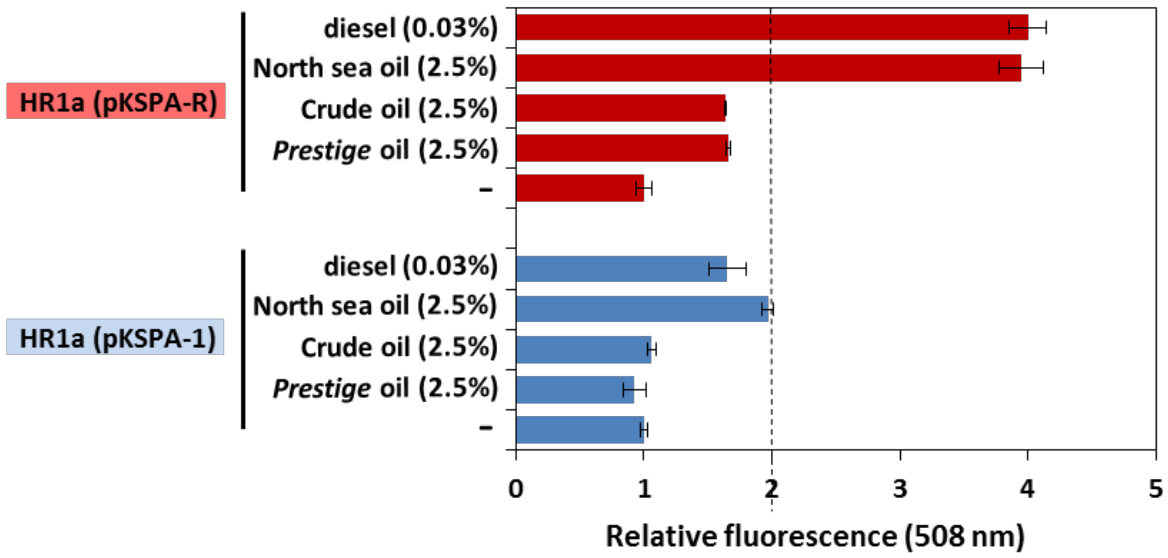

Figure 3 **Comparison of the pKSA-1 and pKSA-R biosensors efficiency with environmental samples.** *Novosphingobium* sp. HR1a bearing either pKSA-1 or pKSA-R biosensor were incubated in the presence of the indicated amounts of hydrocarbon containing solutions. Fluorescence was measured after 5 hours of incubation.

2.2 The TodS/TodT system.

The core of this sensor are the *Pseudomonas putida* TodS-TodT regulatory system and the TodST-responsive P_{todX} promoter fused to the gene coding for the green fluorescent protein (GFP). The TodS-TodT pair (TodST) responds to the presence of toluene or xylene by inducing transcription from the P_{todX} promoter, leading to the synthesis of GFP. TodS is a sensor kinase that ultimately controls *tod* gene expression through its cognate response regulator, TodT. These elements have been cloned into a broad-host range plasmid vector to obtain the pKST1 plasmid, where expression of the *todST* operon is controlled by the benzoate responsive XylS protein through the Pm promoter (Figure 4 see deliverable D2.4). Thus, the biosensor pKST-1 is able to detect BETX compounds due to the capacity of the TodS protein to recognize these monocyclic aromatic compounds. This recognition event occurs at a hydrophobic binding pocket in the protein, where two phenylalanine and one isoleucine residues (F46, I74 and F79) play an important role. Once toluene (or any other BETX compound) binds at this pocket, TodS is auto-phosphorylated and this event leads to the phosphorylation of the DNA-binding protein TodT. The phosphorylated form of TodT is then able to bind the P_{todX} promoter and induce the expression of the GFP protein (Figure 4).

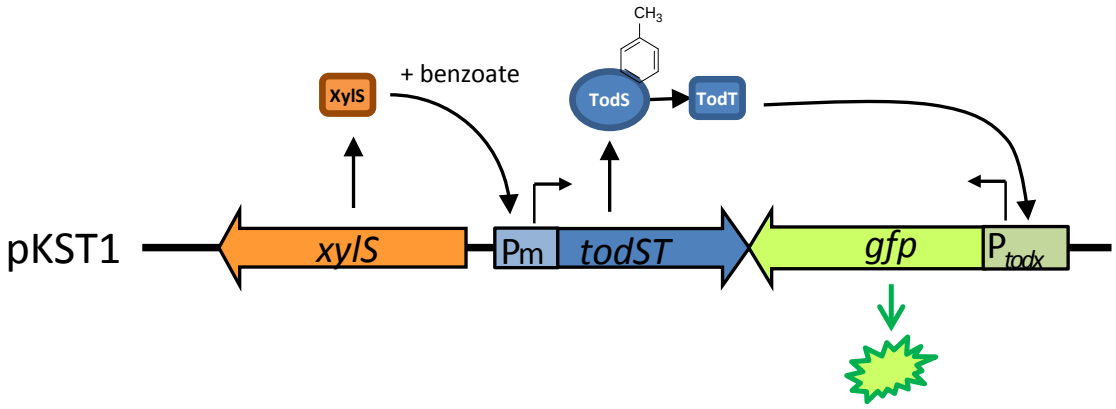

Figure 4 **Scheme of the biosensor for monocyclic aromatic hydrocarbons (toluene/xylene).** The pKST1 plasmid bears the elements required to sense toluene and derivatives in the pSEVA438 broad-host-range vector. The XylS protein synthesized from a constitutive promoter becomes activated by the presence of benzoate in the medium and activates the *Pm* promoter to produce the TodS-TodT protein pair (TodST), which responds to the presence of toluene or xylene by inducing transcription from the *PtodX* promoter, leading to the synthesis of GFP.

2.3 Construction of TodS*-based biosensors: selection of mutant TodS variants

Because the architecture of the TodS binding pocket is the central element in effector recognition, we anticipated that the capacity of TodS to identify larger molecules such as PAHs would require changes in the binding pocket leading to an enlargement of its structure. In order to test the possibility to change the effector specificity of TodS towards PAHs, we selected mutants in the amino acid residues that are involved in the recognition of the BTEX compounds. The three key amino acid residues in the binding site were changed to alanine, resulting in the F46A, I74A and F79A TodS* variants. These mutants showed an altered recognition pattern when exposed to toluene, benzene, xylene and other derivatives (Busch *et al.*, 2007)[1]. Our hypothesis was that these mutants, which bind BETX compounds with affinities different to those of the wild-type, and that are compromised in their ability to activate the P_{todX} promoter with these effectors, might recognize polycyclic aromatic compounds (PAHs). We constructed pSKT-1 derivatives bearing the F46A (pKST-1-46), I74A (pKST-1-74) and F79A (pKST-1-79) mutations in TodS. These plasmid derivatives were introduced into *P. putida* KT2440, and the resulting strains were exposed to toluene, naphthalene, and 2-methyl-naphthalene. Unfortunately, none of these new biosensors were able to detect the presence of PAHs in the medium. Furthermore, they showed no or only a low induction level of green fluorescence in the presence of toluene as compared to pKST-1, which carries the wild type version of TodS.

2.4 Conclusions

In summary, we have developed an improved bioreporter strain that responds to PAHs with high efficiency, both as pure chemicals and in complex mixtures. Although we were not able to engineer TodS response regulator variants able to recognize PAHs, we believe there are currently enough reporter strains available that respond to PAHs, the performance of which can be tested in field assays with different substrates.

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[1] Busch A, Lacal J, Martos A, Ramos JL, Krell T. (2007) Bacterial sensor kinase TodS interacts with agonistic and antagonistic signals. Proc Natl Acad Sci U S A. 104:13774-9