Marine hydrocarbonoclastic bacteria as whole-cell biosensors for n-alkanes

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Summary
Whole-cell biosensors offer potentially useful, cost-effective systems for the in-situ monitoring of seawater for hydrocarbons derived from accidental spills. The present work compares the performance of a biosensor system for the detection of alkanes in seawater, hosted in either Escherichia coli (commonly employed in whole-cell biosensors but not optimized for alkane assimilation) or different marine bacteria specialized in assimilating alkanes. The sensor system was based on the Pseudomonas putida AlkS regulatory protein and the PaikB promoter fused to a gene encoding the green fluorescent protein. While the E. coli sensor provided the fastest response to pure alkanes (25-fold induction after 2 h under the conditions used), a sensor based on Alcanivorax borkumensis was slower, requiring 3–4 h to reach similar induction values. However, the A. borkumensis sensor showed a fourfold lower detection threshold for octane (0.5 µM), and was also better at sensing the alkanes present in petrol. At petrol concentrations of 0.0125%, the A. borkumensis sensor rendered a sevenfold induction, while E. coli sensor showed no response. We discuss possible explanations to this behaviour in terms of the cellular adaptations to alkane uptake and the basal fluorescence produced by each bacterial strain, which was lowest for A. borkumensis.

Introduction
Marine environments face frequent challenges from spills of crude oil or its derivatives, the consequence of accidents that occur during their offshore extraction, transportation or use. Very large-scale accidents, such as those involving the Exxon-Valdez and Prestige oil tankers, or the recent, prolonged spill that occurred following the explosion of the Deepwater Horizon oil platform, are fortunately infrequent, but when they do occur their ecological and economic consequences can be severe (Bragg et al., 1994; Peterson et al., 2003; Albaiges et al., 2006; Bernabeu et al., 2009; Lubchenco et al., 2012; Ryerson et al., 2012). Small-scale spills of crude oil and fuels, which are much more common, have a cumulative effect that can also deteriorate aquatic and marine ecosystems. Efficient, cost-effective systems that allow the in-situ monitoring of such environments for hydrocarbon contaminants derived from crude oil are therefore required (Kalogerakis et al., 2015). Gas chromatography coupled to mass spectrometry (GC-MS) and high-pressure liquid chromatography (HPLC) are the main analytical methods used to test for the presence of hydrocarbons in water samples. Though accurate, these techniques require heavy, complicated and expensive equipment. Biosensors might provide an easy-to-use, fast and cost-effective complementary tool that, while not as accurate as the latter methods, could be very useful for the rapid detection of hydrocarbons in water (Diplock et al., 2010; van der Meer and Belkin, 2010). Biosensors are analytical devices based on biological components, such as whole cells or enzymes that sense a signal and generate a detectable and quantifiable response. An important difference between biosensors and techniques such as GC-MS is that the former respond to the bioavailable fraction of a pollutant, i.e. that available to the cells or to the enzyme used as the sensing device. In contrast, the traditional methods detect the total amount of solvent-extractable pollutant in a sample. Part of this, however, might be unavailable to cells, for example if the polluting molecules are adsorbed onto solids. Differentiating the bioavailable from the non-bioavailable fractions of a contaminant is important when trying to determine its ecotoxicity (Tecon and van der Meer, 2008; Tecon et al., 2009).

Several whole-cell biosensors have been reported that respond to different hydrocarbons (Sticher et al., 1997; Alkasrawi et al., 1999; Stiner and Halverson, 2002; Phoenix et al., 2003; Tecon et al., 2006; 2009; Kumari et al., 2011; Reed et al., 2012; Zhang et al., 2012).

Funding Information This work was funded by the European Commission VII Framework Programme (grant number 312139).

Microbial Biotechnology (2015) 8(4), 693–706
doi:10.1111/1751-7915.12286

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Although they perform well under laboratory conditions, these (and others) suffer a number of problems that limit their use (van der Meer et al., 2004; Harms et al., 2006). For example, the detection of hydrocarbons in water samples is particularly challenging given their low solubility in this medium; this leads to mass transfer problems that limit the availability of hydrocarbons to the cell (Bosma et al., 1997; Jaspers et al., 2001). This can substantially increase the response time of the biosensor and can lead to low signal to noise ratios. One way of improving the reliability of biosensors may be to use as hosts for the sensing systems cells that are adapted for the uptake and metabolism of hydrocarbons. However, a systematic comparison of the performance of a sensor system for hydrocarbons in different bacterial hosts has not been reported to our knowledge.

In recent years, a number of marine bacteria have been described that can efficiently colonize seawater contaminated with hydrocarbons, and that use many hydrocarbons as carbon sources. These bacteria may therefore play an important role in attenuating pollution (Harayama et al., 1997; Panke et al., 1999). Promoter PalkB was fused to a variant of the gfp gene designed for prokaryotic transcriptional fusions and which contains the S65T ‘red shift’ and F64L ‘protein solubility’ mutations (Miller and Lindow, 1997). The alkS gene and the PalkB-gfp fusion were cloned into the broad-host-range plasmid pSEVA431 (Silva-Rocha et al., 2013), thus obtaining plasmid pKSB1 (see Fig. 1). Plasmid pSEVA431 bears the origin of replication of the broad-host-range plasmid pBBR1, and was selected since it was found to replicate in all the bacterial strains examined in the present work. Other vectors based on the replication origin of the broad-host-range plasmid RK2 did not replicate in any of the marine strains tested.

The reporter plasmid pKSB1 was transferred to E. coli W3110 (Jishage and Ishihama, 1997) and to the marine hydrocarbonoclastic bacteria Marinobacter hydrocarbonoclasticus VT8 (Gauthier et al., 1992), A. borkumensis SK2 (Yakimov et al., 1998), Thalassolituus oleivorans Mil-1 (Yakimov et al., 2004), Oleiphilus messinensis ME102 (Golyshin et al., 2002) and Cycloclasticus sp. ME7 (a Mediterranean variant of Cycloclasticus pugetii; Dyksterhouse et al., 1995). The plasmid was successfully mobilized from E. coli to the marine bacteria by conjugation, except for Cycloclasticus sp. ME7, for which electroporation was required (see Experimental procedures). The plasmid was able to replicate in all the bacteria tested. Growth of the Oleiphilus strain was however very slow both in the absence or presence of the plasmid, and only very low turbidity values were reached. The marine strains were cultivated in the artificial seawater medium ONR7a (Dyksterhouse et al., 1995), while E. coli was routinely propagated in M9 mineral salts medium.

**Results**

**Construction of a set of reporter strains containing plasmid pKSB1**

The core of the sensing device used in this work was the AlkS transcriptional activator from the *Pseudomonas putida* OCT plasmid, and the AlkS-responsive PalkB promoter fused to the gene coding for the green fluorescent protein (GFP). The AlkS protein activates transcription from promoter PalkB in the presence of alkanes with 6–10 carbon atoms (Grund et al., 1975; Sticher et al., 1997; Panke et al., 1999).
Table 1. Characteristics of the strains used as hosts for the alkane biosensor.

| Strain** | Marinobacter | Alcanivorax | Thalassolutius | Oleiphilus | Cycloclasticus | E. coli |
|----------|--------------|-------------|---------------|-----------|---------------|--------|
| Growth medium | ONR7a | ONR7a | ONR7a | ONR7a | ONR7a | M9 |
| Carbon sourceb | None | 1% Pyruvate | 1% Pyruvate | 2.5% C14 | 2.5% C14 | 0.25% Naphthalene | 0.4% Glucose |
| (for growth) | | C14 vapours | C14 vapours | C14 vapours | C14 vapours | – | – |
| Carbon sourceb | None | 1% Pyruvate | 1% Acetate | 1% Tween-20 | 0.25% Naphthalene | 0.4% Glucose |
| (conjugation assays) | | | | | | |
| Carbon sourceb | None | 1% Pyruvate | 1% Acetate | 1% Tween-20 | 0.25% Naphthalene | 0.4% Glucose |
| (reporter assays) | | | | | | |
| Growth temperature | 30°C | 30°C | 25°C | 25°C | 25°C | 37°C |
| Doubling time | 78 ± 2 min | 198 ± 2 min | 1445 ± 11 min | NDc | 211 ± 5 min | 73 ± 1 min |
| MIC (solid)d | 32 μg/ml | 16 μg/ml | 2 μg/ml | 1 μg/ml | 12 μg/ml | 1.5 μg/ml |
| MIC (liquid)e | 32 μg/ml | 32 μg/ml | 3 μg/ml | 1 μg/ml | 24 μg/ml | 1 μg/ml |
| Sm’ mutantsa | < 10⁻⁶ | < 10⁻⁶ | < 10⁻³ | < 10⁻⁶ | < 10⁻⁶ | < 10⁻⁶ |

a. The strains analysed were M. hydrocarbonoclasticus VT8, A. borkumensis SK2, T. oleivorans Mil-1, O. messinensis ME102, Cycloclasticus sp. ME7 and E. coli W3110.
b. Percentage carbon sources, given as v/v for C14 and Tween-20, and as w/v for all other compounds.
c. Minimum inhibitory concentration for streptomycin in solid (agar plates) or liquid media.
d. Number of spontaneous mutants resistant to streptomycin appearing on agar plates after 15 days.
e. ND, not-determined. Oleiphilus reached a maximum turbidity (A600) of 0.2 when cultivated in liquid medium with C14 as the carbon source.

(Sambrook and Russell, 2001). Cultivation of E. coli in seawater ONR7a was only possible after a previous acclimatization period, although growth speed decreased to about a half of that detected in M9 medium. Table 1 summarizes the growth conditions and characteristics of the strains used as hosts for the biosensor system.

Time-dependent response of the reporter strains to different alkanes

Preliminary assays showed that, when cultivated in the absence of alkanes, the stationary phase cultures of all the strains analysed showed significant fluorescence at the excitation/emission wavelengths characteristic of the GFP protein, which could compromise the reporter assays. To analyse whether background fluorescence was due to a basal expression of the gfp gene in the absence of alkanes, or to compounds produced and perhaps secreted by the cells, fluorescence measurements were made with cells containing or lacking the reporter plasmid pKSB1, cultivated in their appropriate minimal salts medium (see legend to Fig. 2) and collected at mid-exponential to stationary phase (A600 of 0.5) or in stationary phase (A600 of 1 except for E. coli, that reached an A600 of 3). To determine whether fluorescence was due to compounds expelled to the medium, cells were spun down and re-suspended in fresh medium and fluorescence measured both in the re-suspended cells and in the supernatant. As shown in Fig. 2, all strains showed significant background fluorescence, particularly in stationary phase cultures, irrespective of the absence or presence of the reporter plasmid pKSB1, cultivated in their appropriate minimal salts medium (see legend to Fig. 2) and collected at mid-exponential to stationary phase (A600 of 0.5) or in stationary phase (A600 of 1 except for E. coli, that reached an A600 of 3). To determine whether fluorescence was due to compounds expelled to the medium, cells were spun down and re-suspended in fresh medium and fluorescence measured both in the re-suspended cells and in the supernatant. As shown in Fig. 2, all strains showed significant background fluorescence, particularly in stationary phase cultures, irrespective of the absence or presence of the reporter plasmid in the cells. In E. coli, this fluorescence was much higher in the culture supernatants than in the cells, suggesting that it maybe derived to a large extent from compounds released to the culture medium.

The presence of the reporter plasmid increased the fluorescence emitted by the cells by fourfold, although fluorescence was much lower than that observed in the culture supernatants, suggesting that there is some basal expression of GFP in the absence of alkanes, although its contribution to the overall fluorescence is less than 25%. The cells of A. borkumensis showed the lowest background fluorescence, and most of it derived from compounds present in the culture supernatants. The values observed for M. hydrocarbonoclasticus and T. oleivorans were in most cases midway between those of E. coli and A. borkumensis. The contribution of the basal expression of GFP to the overall background fluorescence was substantial in both M. hydrocarbonoclasticus and T. oleivorans, although a significant fluorescence was also detected in culture supernatants irrespective of the presence or absence of the reporter plasmid.

To minimize the problem of background fluorescence in the bioreporter assays, these were performed with cells that were previously centrifuged and re-suspended in fresh medium containing the appropriate carbon source for each strain (glucose, pyruvate or acetate, see Table 1). However, the Marinobacter strain quickly developed a strong fluorescence signal once again, despite there being no hydrocarbons present that could induce GFP production. This was reduced if the fluorescence assays were performed with the Marinobacter cells re-suspended in a fresh medium containing no carbon source.

Different pure n-alkanes (hexane, octane, decane, dodecane, tetradecane, hexadecane, octadecane and eicosane, abbreviated hereafter as C6, C8, C10, C12, C14, C16, C18 and C20, respectively) were added to the cells at a concentration of 1%, which is above their solubility limit in water. A control assay involving no alkane was performed in parallel. An alkane-only control, lacking cells,
was also included. The samples were incubated at 30°C with agitation in closed glass vials limiting the headspace to 10% of the total volume. At different times, the fluorescence was measured as indicated in Experimental procedures, normalizing the values observed relative to the culture turbidity. In the absence of \( n \)-alkanes, the normalized fluorescence observed after 2 h for the reporter strains containing plasmid pKSB1 was lowest for \textit{A. borkumensis} (920 ± 60 units), intermediate for \textit{E. coli} (6770 ± 715 units), and significantly higher for the rest of the strains (9710 ± 1180 units for \textit{T. oleivorans}, 9715 ± 120 units for \textit{O. messinensis}, 10530 ± 385 units for \textit{Cycloclasticus} sp. ME7 and 10860 ± 85 units for \textit{M. hydrocarbonoclasticus}). As shown in Fig. 3, the \textit{E. coli}, \textit{Marinobacter}, \textit{Alcanivorax} and \textit{Thalassolituus} strains containing plasmid pKSB1 showed a good response to \( C_9, C_{12} \) and \( C_{16} \) alkanes. The response was already clear after 1 h, and increased steadily over time. No response was obtained with alkanes of 11 or more carbon atoms. This agrees with previous reports that, at least in \textit{E. coli}, the AlkS/PalkB system responds only to \( C_6-C_{10} \) alkanes (Sticher et al., 1997; Reed et al., 2012). The \textit{E. coli} strain responded faster than the others; in fact the assay was stopped after 2 h since at longer incubation times the fluorescence signal saturated the detector. If the background signal obtained in the absence of alkanes was subtracted to that obtained in the presence of alkanes, the response of the \textit{E. coli} biosensor after a 2 h incubation was about fivefold higher than that of the other reporter strains. However, allowing the bioreporter
assay to proceed for 4 h led to a clear improvement in the response of the *Marinobacter*, *Alcanivorax* and *Thalassolituus* strains, with little increase in the background signal (Fig. 3). The *E. coli* cells seemed to be sensitive to the ionic strength of the incubation medium, since the alkane response was very good when cells were incubated in the M9 mineral salts medium but became undetectable when the assay was performed in the artificial seawater medium ONR7a, which has a much higher ionic strength (Fig. 3A). The sensitivity of *E. coli* bioreporters to seawater has been observed previously (Tecon et al., 2010); these authors indicated that seawater samples had to be diluted at least four times to avoid inhibition of the reporter assays by the salt. As an alternative approach, prior to the reporter assays *E. coli* cells were cultivated directly in ONR7a medium, conditions in which growth is possible after an acclimatization period, although growth rate decreases significantly. However, the response to alkanes of *E. coli* cells adapted to grow in ONR7a medium was about four to sixfold lower than that of cells cultivated in M9 medium, and the variability of the assays was higher (not shown).

When considering the fluorescence induction ratios (signal observed in the presence of alkanes divided by that observed in its absence), the background fluorescence in the absence of alkanes had an important impact. Induction of fluorescence was strong in the *Alcanivorax* (around 50-fold that seen in the absence of alkanes for C6 after 4 h) and *E. coli* (around 25-fold after 2 h) strains, and clearly lower (six to eightfold) in the *Marinobacter* and *Oleiphilus* strains. The response to alkanes of *E. coli* cells adapted to grow in ONR7a medium was about four to sixfold lower than that of cells cultivated in M9 medium, and the variability of the assays was higher (not shown).

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Marinobacter and Thalassolituus strains (Fig. 4). The E. coli and Marinobacter strains showed a similar response, irrespective of the chain length of the alkanes, while the Alcanivorax strain responded better to C6 than to C10. The Thalassolituus strain showed the opposite behaviour (Figs. 3 and 4). This suggests differences in the way in which each of these bacterial strains gains access to the different alkanes.

Detection threshold of the reporter strains to octane

The sensitivity and linearity of the reporter strains’ response to octane were determined by fluorescence assays in the presence of increasing alkane concentrations (0.2 μmol/L, 0.5 μmol/L, 1 μmol/L, 2 μmol/L, 5 μmol/L, 10 μmol/L, 25 μmol/L and 50 μmol/L). All assays were conducted for 2 h in triplicate, and the data analysed by one-way analysis of variance. The Marinobacter, Alcanivorax and Thalassolituus strains were able to detect octane concentrations as low as 0.5 μM with statistical significance (Fig. 5B–D). However, the E. coli strain did not respond to octane concentrations lower than 2 μM (Fig. 5A). For the E. coli, Marinobacter and Alcanivorax strains, the response was linear up to 5 μM octane ($R^2 > 0.99$); at higher octane concentrations the signal increase declined, and little or no further increase was observed above 10 μmol/L octane (Fig. 6). This is consistent with the water solubility limit of this hydrocarbon (about 6.3 μM). For the Thalassolituus strain, loss of linearity started at octane concentrations of 2.5 μM.

Response of reporter strains to water contaminated with petrol or crude oil

Due to their hydrophobicity, alkanes have a strong tendency to partition into organic solutes such as petrol (gasoline) or crude oil. This could reduce the efficiency of biosensors designed to detect hydrocarbons in contaminated seawater. The behaviour of the present biosensor strains when confronted with water containing petrol or crude oil was investigated in the same way as for the pure alkanes. The petrol used, obtained from a commercial filling station, contained significant amounts of C7 (121 mM) and C8 (38 mM) alkanes, and smaller amounts of C9 (5.2 mM) and C10 (0.9 mM) alkanes (Fig. 7A). Toluene, xylene and trimethylbenzene were abundant. The total linear short-chain alkanes in the petrol reached a concentration of 165 mM. However, when added to the water samples in the bioreporter assays, the petrol was diluted by more than three orders of magnitude, bringing the n-alkane concentration into the micromolar range.

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Different final concentrations of petrol (0.1%, 0.05%, 0.025% and 0.0125% v/v) were added directly to the M9 mineral salts medium for the E. coli strain, or the ONR7a medium for the Marinobacter, Alcanivorax and Thalassolitus strains. The sample was vortexed to ensure mixing, and then added to the medium containing the reporter strains. A clear fluorescence signal was detected after 2 h for all four strains (Fig. 7B). At the highest concentration of petrol used, the response of the Alcanivorax reporter almost doubled that of the E. coli strain, while the response of the Marinobacter and Thalassolitus strains was much poorer, in part due to the high fluorescence recorded in the absence of the inducer, which reduced the final fluorescence induction ratio. When added at 0.05%, the Alcanivorax strain returned induction values about three to four times higher than those of the other strains. At petrol concentrations of 0.025% or lower, only the Alcanivorax strain rendered a significant response (Fig. 7B).

The response of the Alcanivorax and E. coli reporter strains to crude oil was also analysed. For this, a Dansk blend crude oil (a mixture of crude oils from multiple fields in the Danish sector of the North Sea) was used. Gas chromatography coupled to mass spectrometry chromatography analysis of this oil blend showed it to have very low or undetectable amounts of n-alkanes shorter than 12 carbon atoms (data not shown). Crude oil was added directly to the fluorescence assays at a final concentration of 0.1% (v/v). As predicted from the lack of C6-C10 n-alkanes in the oil, neither the E. coli nor the Alcanivorax reporter strains showed any clear response. However, when the oil was previously spiked with a mixture of C6-C14 n-alkanes (7% each, final concentration), a clear response was observed for both strains, the fluorescence induction values being close to 15-fold in both cases (Fig. 8). This is consistent with the high amount of alkanes added to the crude oil. The E. coli strain showed a good fluorescence response only if the assay was performed in
Discussion

The aim of the present work was to compare the performance of a biosensor system for n-alkanes when introduced into different bacterial strains highly – or not at all – specialized in the degradation alkanes. The hope was that the specialized alkane degraders, which might be expected to have optimized systems for the uptake of n-alkanes, would react faster and more efficiently than non-specialized bacteria to the presence of these compounds at low concentration in seawater samples. The eventual goal was to tackle one of the limitations of currently available biosensors for hydrocarbons, namely the low mass transfer of the hydrocarbon from the water phase to the sensing cells. This is believed to reduce the sensitivity and speed of response of these analytical devices (Bosma et al., 1997; van der Meer et al., 2004; Tecon and van der Meer, 2008; Diplock et al., 2010). The biosensor system used was based on the n-alkane-responsive AlkS transcriptional activator from the P. putida OCT plasmid and the fusion of the AlkS-activated PalkB promoter to the gfp gene.

All strains showed detectable background fluorescence in the absence of alkanes, even if cells lacked the reporter plasmid. Fluorescence was significantly higher in stationary phase cells than in exponentially growing cells, and was stronger for the E. coli strain and lowest for the Alcanivorax strain, the Marinobacter and Thalassolituus strains showing intermediate background levels. This problem was to a large part due to the accumulation of fluorescent compounds in the growth medium, and could be significantly reduced by spinning down the cells and resuspending them in fresh medium before the biosensor assays.

The E. coli reporter showed a rapid, strong response to C₆-C₁₀ alkanes provided that the assay was performed in a mineral salt medium of moderate ionic strength, such as M9. When the hydrocarbons were provided in ONR7a medium, which resembles seawater, E. coli cells that had not been pre-adapted to seawater were unable to respond. Pre-adaptation of E. coli to the high ionic strength ONR7a medium, which should trigger an osmotic stress response (Hengge-Aronis, 1996), allowed cells to
respond to alkanes, although the signal was significantly weaker and less reproducible than when the M9 medium was used. This sensitivity of *E. coli*-based biosensors to seawater has been observed previously, and could be solved by diluting the seawater samples by at least four-fold (Tecon *et al.*, 2010). Under optimal conditions – cells grown and assays performed in M9 medium – induction of fluorescence in the *E. coli* biosensor was very good, reaching values about 25-fold higher than the background after 2 h when C6, C8 or C10 alkanes were provided at concentrations above their solubility limits. The *Marinobacter*, *Alcanivorax* and *Thalassolituus* reporter strains, all specialized alkane degraders, provided slower and less intense responses than *E. coli*, although the normalized fluorescence detected after 2–4 h was high.

Nevertheless, since the *Marinobacter* and *Thalassolituus* strains showed high background fluorescence levels in the absence of alkanes, the final induction ratios after 2–4 h were in the range of three to eightfold. In part due to its low background fluorescence, the *Alcanivorax* strain showed very high induction ratios, with values of 12 to 14-fold after 2 h, and of up to 50-fold (for C6), 23-fold (for C8) and 16-fold (for C10) after 4 h. These very high values make this strain a very good host for a whole-cell biosensor designed to detect C6–C10 *n*-alkanes in seawater samples. In this same line, *A. borkumensis* was also found useful to analyse the bioavailability of long-chain alkanes on the basis of a related reporter system sensitive to alkanes of more than 13 carbon atoms (Kumari *et al.*, 2011).

Under the experimental conditions used, the lowest concentration of C8 to return a clear and statistically significant induction signal was about 0.5 μM for the three marine strains, and 2 μM for the *E. coli* strain. These values are below the solubility limits of C8 in water (about 6.3 μM). The linearity of the response was lost at octane concentrations close to and above its solubility limit. Most bacterial reporter assays for hydrocarbons can detect the target compounds in the nanomolar to micromolar range (reviewed in van der Meer *et al.*, 2004), the response depending to a large extent on the experimental conditions and the reporter system used.

The usefulness of the reporter strains when confronted with water samples containing commercial petrol or crude oil was also analysed. For the petrol, which contained significant amounts of C7-C9 *n*-alkanes, the *Alcanivorax* and the *E. coli* strains provided the best results, returning...
induction values in the range of 25-fold (for Alcanivorax) or 14-fold (for E. coli) just 2 h after adding petrol at 0.1% (v/v). The Alcanivorax strain was clearly superior to E. coli in detecting lower petrol concentrations, providing fluorescence induction ratios of sevenfold at petrol concentrations as low as 0.012%, while E. coli was barely able to respond at petrol concentrations of 0.025% and rendered no signal at a concentration of 0.012%. The performance of the Marinobacter and Thalassolituus strains was again compromised by the high background fluorescence observed in the absence of petrol.

The response to crude oil was tested only with the E. coli and Alcanivorax reporter strains. The crude oil initially used, which was devoid of n-alkanes of less than 12 carbon atoms, was essentially unable to induce a response in either strain. Spiking the oil with a mixture of C6-C14 n-alkanes prior to the reporter assay did induce a strong response in both strains (13- to 16-fold). This shows the excellent specificity of the biosensor, which only detects C6-C10 alkanes; no other oil hydrocarbons induced a signal. Alkanes with between 6 and 10 carbon atoms volatilize rapidly after an oil spill, and their concentration in contaminated seawaters decreases significantly after 2–3 days (Tecon et al., 2010). However, they are more toxic than higher molecular weight alkanes due to their greater water solubility, and hence greater bioavailability. Monitoring C6-C10 alkanes in contaminated water samples is therefore important.

For detecting n-alkanes of relatively low molecular weight, both the non-specialized alkane degrader E. coli and highly specialized alkane degraders such as A. borkumensis, are good hosts for the AlkS/PalkB-gfp biosensor derived from the P. putida OCT plasmid. However, the usefulness of E. coli as a host for this sensing system was compromised by its salt sensitivity and its lower performance when confronted to low hydrocarbon concentrations. Alcanivorax borkumensis, a marine bacterium specialized in degrading alkanes, showed threshold detection levels about fourfold lower than those of E. coli. In addition, it showed background fluorescence levels in the absence of alkanes significantly lower than those of E. coli. Therefore, the better performance of A. borkumensis is likely due to a combination of reasons. It is noteworthy that the Marinobacter and Thalassolituus marine strains also showed fourfold lower detection thresholds for C8 than the E. coli reporter. The specialization in alkane degradation of the three marine strains may perhaps contribute to their low detection thresholds, possibly because they have evolved efficient uptake systems for these highly hydrophobic molecules. However, little is known about how bacterial cells internalize water-insoluble hydrocarbons. The outer membrane of Gram-negative bacteria is an effective permeability barrier to them. Some strains that can use these compounds as a carbon source are endowed with porins that facilitate, for example, the passage of alkanes (van Beilen et al., 2001; Julsing et al., 2012; Grant et al., 2014; Wang and Shao, 2014) or toluene (Kahng et al., 2000; Kasai et al., 2001; Hearn et al., 2008) through the outer membrane. Thereafter, it is unclear whether they cross the inner membrane using active transport systems or whether they simply diffuse through the hydrophobic lipid bilayer.

The present results highlight the importance of choosing bacterial strains with low background fluorescence values in the absence of an inducer as hosts for biosensor systems based on the GFP. While backgrounds derived from the excretion of fluorescent molecules can be avoided by centrifuging the cells prior to their use in assays, those caused by a high basal expression of the PalkB-gfp fusion cannot be easily avoided; a host with low basal expression is therefore vital. Among the strains tested, A. borkumensis showed the lowest background fluorescence. In summary, its adaptation to seawater, its low background fluorescence and its superior ability to detect alkanes at low concentrations makes A. borkumensis the best host possible — among those tested – for the present reporter system when attempting to detect C6-C10 alkanes in seawater.

**Experimental procedures**

**Bacterial strains and culture media**

The strains used in this work were E. coli W3110 (Jishage and Ishihama, 1997), E. coli DH5α (Woodcock et al., 1989), E. coli HB101 (pRK600) (Kessler et al., 1992), M. hydrocarbonoclasticus VT8 (Gauthier et al., 1992), A. borkumensis SK2 (Yakimov et al., 1998), T. oleivorans Mil-1 (Yakimov et al., 2004), O. messiniens ME102 (Golyshin et al., 2002) and Cycloclasticus sp. ME7 (a Mediterranean variant of C. pugetii) (Dyksterhouse et al., 1995). Unless otherwise stated, E. coli strains were grown at 37°C in complete LB medium, or in M9 mineral salts medium supplemented with 0.4% (w/v) glucose and 0.1% (w/v) thiamine (Sambrook and Russell, 2001). The marine strains were cultivated in the artificial seawater mineral salts medium ONR7a, which is based on the ionic composition of seawater (Dyksterhouse et al., 1995). Marinobacter and Alcanivorax strains were cultivated at 30°C using 1% (w/v) pyruvate or 2.5% (w/v) tetradecane as the carbon source. Thalassolituus and Oleiphilus strains were cultivated at 25°C using 2.5% (w/v) tetradecane as the carbon source; where indicated, 1% acetate or 1% Tween-20, respectively, were used instead. Cycloclasticus strains were cultivated at 25°C using 0.25% (w/v) naphthalene as the carbon source.

**Determination of sensitivity to streptomycin**

To determine the sensitivity of the hydrocarbonoclastic marine strains to streptomycin (the antibiotic used to select for plasmid pKSB1), cells were cultivated to stationary phase in ONR7a medium with the appropriate carbon source (see
Table 1), centrifuged and re-suspended in the same culture medium to a turbidity (A600) of 0.1. One hundred μl of each culture were plated onto an ONR7a medium agar plate containing the carbon source of choice, and the cells allowed to adsorb onto the plate for 30 min at room temperature. A streptomycin e-test strip (0.064 to 1024 μg/ml; Biomerieux, France) was equilibrated for 30 min at room temperature and then applied onto the agar surface. Cells were allowed to grow until a lawn was visible. The minimum inhibitory concentration (MIC) was considered to be the point on the strip scale where growth inhibition began to be evident. Tests were performed in triplicate. Using the values obtained as a reference, the assays were repeated in test tubes containing liquid medium. See Table 1 for results.

Construction of plasmid pKSB1

A DNA fragment containing the alkS gene (including its own promoter) coding for the alkane-responsive AlkS transcriptional regulator was excised from plasmid pTS1 (Yuste et al., 1998) with HindIII and BsaAI restriction endonucleases, and inserted between the HindIII and Smal sites of plasmid pSEVA 424 (Silva-Rocha et al., 2013) to render plasmid pKSalkS. In parallel, a DNA segment was constructed in which the gfp gene is transcribed from promoter PalkB, i.e. the AlkS-regulated promoter for the genes of the alkane degradation pathway encoded in the P. putida OCT plasmid (Kok et al., 1989; Panke et al., 1999; Canosa et al., 2000). The gfp gene was excised from pGreenTIR (Miller and Lindow, 1997) as an EcoRI DNA fragment and the ends blunted with T4 DNA polymerase before cloning it into the KpnI site (blunted with T4-DNA polymerase) of plasmid pPB7 (Yuste et al., 1998) under the influence of promoter PalkB. The plasmid obtained was named pPBG1. The PalkB-gfp transcriptional fusion was excised from pPBG1 with HindIII and SacI, and cloned between the same restriction sites of pUC18Not, yielding plasmid pPBG2. The PalkB-gfp fusion was extracted from pPBG2 with NotI and cloned at the HindIII site of pKSalkS, after blunting the ends of both fragments with T4 DNA polymerase. A plasmid was selected in which the alkS gene and the PalkB-gfp fusion were in the opposite orientation (named pKSC1). Since this plasmid was found to be unable to replicate in the marine strains used, a DNA fragment including alkS and PalkB-gfp was excised from it using NotI sites that flanked the DNA fragment (sites that derive from the plasmid vector used), and the DNA segment obtained was cloned into the NotI site of the broad-host-range plasmid pSEVA 431 (Silva-Rocha et al., 2013), obtaining plasmid pKSB1.

Conjugation assays

Plasmid pKSB1 was introduced into the Marinobacter, Alcanivorax, Thalassolituus and Oleiphilus strains by tripartite conjugation assays, using E. coli DH5α (pKSB1) as the donor, E. coli HB101 (pRK600) as a helper of transfer functions and the selected marine strain as the recipient. To this end, E. coli strains DH5α (pKSB1) and HB101 (pRK600) were cultivated to stationary phase in LB medium supplemented with 50 μg/ml streptomycin or 50 μg/ml of chloramphenicol, respectively, while the marine strains were cultivated to stationary phase in ONR7a medium with the appropriate carbon source. Cells were centrifuged and re-suspended in 1 ml of cLB (10 g/l of triptone, 5 g/l of yeast extract, 0.45 g/l Na2HPO4 · 2H2O, 2.5 g/l NaNH2O, 11.5 g/l NaCl, 0.38 g/l KCl, 0.7 g/l CaCl2 · H2O and 2% sodium pyruvate; Sabirova et al., 2006) in donor/helper/recipient proportions of 1:2:4. Cells were placed onto a nitrocellulose filter (0.45 μm pore size) and incubated at 25°C for 24 h. They were then collected from the filter in 1 ml of ONR7a, diluted and plated onto ONR7a agar plates with the required carbon source (C6 for Marinobacter and C14 for Alcanivorax, Thalassolituus and Oleiphilus, in all cases applied to the plate lid to saturate the vapour phase) and streptomycin (100 μg/ml for Marinobacter, 50 μg/ml for Alcanivorax, 5 μg/ml for Thalassolituus and 2.5 μg/ml for Oleiphilus). Dicyclopropylketone (a gratuitous inducer that mimics the effect of alkanes; Grund et al., 1975) was also included in the agar plate at a final concentration of 0.05% (v/v) to induce the PalkB promoter and allow the expression of the gfp gene. Plates were incubated at 30°C for Marinobacter and Alcanivorax strains, and at 25°C for Thalassolituus and Oleiphilus strains, until colonies appeared. Colonies showing fluorescence derived from GFP were selected using a luminometer and the presence of plasmid pKSB1 verified by colony polymerase chain reaction (PCR) using the oligonucleotides GFP 300′-AAAGATGACGGGAACATAGAAG-3′ and GFP Rev (5′-GTAGTTATGTGTTATCC-3′), which amplify the gfp gene. The phylogenetic identity of the selected transconjugants was checked by PCR amplification and sequencing of the 16S rRNA gene, using oligonucleotides 16S-8a27D (5′-AGAGTTTGACCTTGGCTCAG-3′) and 16S-1510R (5′-GGTTACCTTGTTAAGCT-3′) as primers.

Electroporation

Plasmid pKSB1 could not be introduced into Cycloclasticus sp. ME7 by conjugation; an electroporation protocol was therefore used instead. Bacteria were plated onto ONR7a with naphthalene and, when colonies were evident, cells were taken with an inoculating loop and re-suspended in water with 20% glycerol. Two hundred ng of plasmid DNA were added, and the mixture was kept on ice for 1 min. It was transferred to an electroporation cuvette (0.1 cm) and a pulse of 1.8 KVP applied in a Micropulser (Biorad). One millilitre of ONR7a was immediately added to the 30 μl, and the mixture transferred to a flask containing 4 ml of ONR7a and 0.25% (w/v) naphthalene as a carbon source. The flask was incubated 4 h at 25°C with agitation. The cells were then plated onto ONR7a plates with 30 μg/ml of streptomycin and naphthalene (provided as vapour from crystals placed on the plate lid) and incubated at 25°C until colonies appeared. The presence of the plasmid was confirmed by PCR using oligonucleotides for the gfp gene.

Fluorescence assays with pure alkanes

A culture of each reporter strain was cultivated to stationary phase in the appropriate medium as specified above (see Bacterial strains and culture media). Cells were centrifuged
and re-suspended in fresh medium with the appropriate concentration of streptomycin to select for plasmid pKSB1. The carbon source added was glucose for _E. coli_, pyruvate for _Alcanivorax_, acetate for _Thalassolituus_, Tween-20 for _Oleiphilus_ and naphthalene for _Cycloclasticus_. For the _Marinobacter_ strain, the assays were performed without a carbon source to minimize autofluorescence and improve the signal/noise ratio in the presence of alkanes. After adjusting the turbidity of the culture to 0.9–1 with fresh medium, 2 ml of the cell suspension were loaded into 4.5 ml crew-cap glass vials (one vial per test). In parallel, different alkanes were added to 2 ml of cell-free culture medium; when hydrocarbons were added at 1%, these were directly supplied to the medium and the vials agitated for 1 min to homogenize the mixture and saturate the water phase with the hydrocarbon. When analysing the concentration-dependent response of the reporter cells, alkanes were added from stock solutions prepared in dimethyl sulphoxide. Two millilitres of the alkane-containing medium, or of alkane-free medium for control reactions, were added to the vial containing 2 ml of the cell suspension; the vials were tightly closed to avoid evaporation of the hydrocarbons and incubated at 30°C with agitation (end-over-end) for 1 to 4 h, as specified. Vials containing growth medium with different inducers, but lacking cells, were used as blanks. For measuring fluorescence, 200 μl samples were taken (in triplicate; technical replicates) from the vial and dispensed onto a black, clear-bottomed microtitre plate. The fluorescence (excitation 480 nm, emission 520 nm) and absorbance (600 nm) were measured simultaneously and the values represented as normalized fluorescence, dividing the fluorescence induc-

...and, after removing the sodium sulphate by centrifugation, the sample was stored at −20°C. Samples were then analysed by GC-MS. For GC-MS analyses, samples were diluted 100-fold and 1 μl of the diluted sample injected into a GC-MS analyser (model Varian 3800) equipped with a Factor VI column (30 m × 0.25 μm × 0.25 mm). Linear alkanes were identified by their retention time and mass spectra, using pure alkanes as standards prepared at 1000 ppm in dichloromethane. Gas chromatography/mass spectrometry analyses were performed at the Research Interdepartmental Service of the Autonomous University of Madrid, Madrid, Spain.

Conflict of interest

None declared.

Acknowledgements

We are grateful to Ana Segura for helpful discussions, to Peter Golyshin and Michail Yakimov for providing the marine bacterial strains used and to the Dansk Underground Consortium for providing the Dansk blend crude oil.

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