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Balseiro-Romero, Maria; GKOREZIS, Panos; Kidd, Petra S.; Van Hamme, Jonathan; WEYENS, Nele; Monterroso, Carmen & VANGRONSVELD, Jaco (2017)
Characterization and degradation potential of diesel-degrading bacterial strains for application in bioremediation. In: INTERNATIONAL JOURNAL OF PHYTOREMEDIATION, 19(10), p. 955-963.

DOI: 10.1080/15226514.2017.1337065
Handle: http://hdl.handle.net/1942/26340

International Journal of Phytoremediation

ISSN: 1522-6514 (Print) 1549-7879 (Online) Journal homepage: http://www.tandfonline.com/loi/bijp20

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To cite this article: María Balseiro-Romero, Panagiotis Gkorezis, Petra S. Kidd, Jonathan Van Hamme, Nele Weyens, Carmen Monterroso & Jaco Vangronsveld (2017): Characterization and degradation potential of diesel-degrading bacterial strains for application in bioremediation, International Journal of Phytoremediation, DOI: 10.1080/15226514.2017.1337065

To link to this article: http://dx.doi.org/10.1080/15226514.2017.1337065

Accepted author version posted online: 09 Jun 2017.

Characterization and degradation potential of diesel-degrading bacterial strains for application in bioremediation

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ABSTRACT

Bioremediation of polluted soils is a promising technique with low environmental impact, which uses soil organisms to degrade soil contaminants. In this study, 19 bacterial strains isolated from a diesel-contaminated soil were screened for diesel-degrading potential, biosurfactant production, and biofilm formation abilities, all desirable characteristics when selecting strains for re-inoculation into hydrocarbon-contaminated soils. Diesel degradation rates were determined *in vitro* in minimal medium with diesel as the sole carbon source. The capacity to degrade diesel range organics (DRO) of strains SPG23 (*Arthobacter* sp.) and PF1 (*Acinetobacter oleivorans*) reached 17-26% of total DRO

after 10 days, and 90% for strain GK2 (*Acinetobacter calcoaceticus*). The amount and rate of alkane degradation decreased significantly with increasing carbon number for SPG23 and PF1. Strain GK2, which produced biosurfactants and biofilms, exhibited a greater extent, and faster rate of alkane degradation compared to SPG23 and PF1. Based on the outcomes of degradation experiments, in addition to biosurfactant production, biofilm formation capacities, and previous genome characterizations, strain GK2 is a promising candidate for microbial-assisted phytoremediation of diesel-contaminated soils. These results are of particular interest to select suitable strains for bioremediation, not only presenting high diesel degradation rates, but also other characteristics which could improve rhizosphere colonization.

KEYWORDS

diesel biodegradation; biosurfactants; biofilm

INTRODUCTION

The accidental release of hydrocarbons into soils, due to spillages in the petrochemical industry, distribution of fuels and leakage from storage tanks, is of great environmental concern because of the toxicity and carcinogenicity of petroleum products [1]. Civil-engineering based remediation methods are usually expensive and have impacts on soil environments that may lead to loss of ecosystem functionality. Soil remediation by biological methods based on the application or biostimulation of hydrocarbon-degrading microorganisms in contaminated soils (bioremediation), or in association with plants (phyto- and rhizo- remediation), is a proven alternative to physicochemical remediation schemes [2–8]. Bioremediation techniques are less expensive and less invasive *in situ* technologies that allow for the conversion of the contaminants into less or nontoxic forms [9].

Petroleum hydrocarbon metabolism by soil microorganisms has been well studied for over a century [10–12], with many bacterial genera described as hydrocarbonoclastic: members of the genera *Bacillus, Pseudomonas, Acinetobacter, Gordonia, Brevibacterium, Aeromicrobium, Dietzia, Burkholderia,* and *Mycobacterium* to name a few. The metabolic pathways of petroleum hydrocarbon degradation have been described as being mediated by genes coding for enzymes such as alkane hydroxylases (*e.g. alkB* related) (found in some members of *Pseudomonas*, *Burkholderia*, *Rhodococcus*), monooxygenases (*e.g.* in *Methylococcus*, *Methylocella*, or *Methylobacter*), and bacterial P450 oxygenase systems (e.g. in *Acinetobacter, Caulobacter* and *Mycobacterium*) [1].

The success of a bioremediation process is related to the metabolic capabilities of the degrading microorganisms [13], as well as to the presence of favourable environmental

conditions (oxygen, pH, nutrients, *etc*.) to guarantee optimal growth and degradation rates [1]. The susceptibility of hydrocarbons to biodegradation depends on the microbial genetic potential, on the properties of the hydrocarbon substrate (*i.e.* hydrophobicity, concentration, volatility, molecular mass, bioavailability, water solubility, *etc*.), and on environmental factors (*i.e.* soil organic matter content, particle size, humidity, *etc.*) [13,14]. Microbial surfactants, or biosurfactants, can exert influence on hydrocarbon-water interfaces, in some cases improving hydrocarbon bioavailability by increasing apparent water solubility reducing interfacial tensions [15,16]. For non-aqueous phase hydrocarbons, biofilms may improve microbial access to hydrophobic surfaces, and improve overall microbial community health as, compared to planktonic microorganisms, cells in biofilms generally exhibit higher metabolic activity and population densities, greater survival (as they are protected from external stresses), and higher rates of gene transfer [17]. Biofilm forming strains are especially suitable for the remediation of recalcitrant compounds, since the ability to immobilize contaminants and the high cell density accelerates the usage of xenobiotics [18].

The aim of the present study was to screen a wide collection of bacterial strains isolated from a diesel-contaminated site in Genk (Belgium) (both from rhizospheric soil and plant tissues) for their diesel-degrading abilities, biosurfactant production and biofilm formation in the presence of hydrocarbons. Furthermore, diesel degradation rates were estimated by *in vitro* incubation in minimal culture medium supplemented with diesel for a selection of strains. The results will be a valuable decision tool to select the best strains to be used as

bioaugmentation inoculants in bio- and phyto- remediation strategies for diesel-contaminated soils.

MATERIALS AND METHODS

Bacterial strains

Nineteen bacterial strains (Table 1) were isolated from a diesel-contaminated site of the Ford Motor Company in Genk (Belgium) (from both rhizospheric soil and plant tissues) and screened for their capacities for diesel-degradation, biosurfactant production and biofilm formation. At this site, hybrid poplar [*Populus deltoides x (trichocarpa x deltoides*) cv. Grimminge] was planted as a means of controlling the contamination plume [19].

From this collection, draft genomes were prepared for *Arthrobacter* sp. SPG23 [20]; *Acinetobacter oleivorans* PF1 [21] and *Acinetobacter calcoaceticus* GK2 (data not published). Whole genome shotgun projects have been deposited at DDBJ/EMBL/GenBank under the accession numbers, JYCN00000000, JHQK00000000 and LQMV00000000, respectively. The versions described in the corresponding genome announcement papers are version JYCN01000000 for SPG23, JHQK01000000 for the PF1 and LQMV01000000 for the GK2. Both SPG23 and GK2 were isolated from poplar rhizospheric soil, and PF1 is an endophyte isolated from poplar tissues. Genes for plant growth-promoting characteristics were present spread throughout the genome, corroborating results from phenotypic tests: PF1 [20] and GK2 (Panagiotis Gkorezis, personal communication, February 2017) have the capacities for 1-aminocyclopropane-1-carboxylate deaminase activity, auxin biosynthesis, siderophore production, and inorganic phosphorous solubilization; and SPG23, for nitrogen

fixation, and also for siderophore production and inorganic phosphorous solubilization and uptake [21]. These strains presented alkane-degrading genes spread across the genome (principally genes for the alkane hydroxylase system), as well as other genes involved in the degradation of aromatic hydrocarbons (benzene or naphthalene). According to these characteristics, these bacterial strains were then selected for the estimation of diesel degradation capacity *in vitro*.

Screening assays for biosurfactant production

Cultures were prepared by growing the strains in Nutrient Broth (NB) medium (per L: 1 g D(+) glucose; 15 g peptone, 6 g NaCl, and 3 g yeast extract) at 37 ºC and 120 rpm for 10-12 h. After incubation, cultures were centrifuged (6000 rpm, 15 min) washed twice and re-suspended to an optical density of 1 at 660 nm (OD_{660nm}) in biosurfactant (BS) production selective medium. Bacterial suspensions at 3% were used to inoculate 50 mL of selective medium in 250-mL Erlenmeyer flasks (*n=3*), and incubated at 37 ºC and 160 rpm for 5 days. The BS production selective medium contained (per L): 10.0 g $(NH_4)_2SO_4$, 1.1 g NaCl, 1.1 g KCl, $2.8 \cdot 10^{-4}$ g $FeSO_4 \cdot 7H_2O$, 4.4 g $K_2HPO_4 \cdot 3H_2O$, 3.4 g KH_2PO_4, 0.5 g $MgSO_4$, and 0.5 g yeast extract. This medium was supplemented with 0.5 mL of filter-sterilized trace element solution (0.2 µm, Millipore) (per L: 0.24 g $CaCl_2$, 0.29 g $ZnSO_4$, 0.17 g $MnSO_4$, and 0.25 g $CuSO_4$), and 2% (v/v) of filter-sterilized diesel (PTFE 0.45 µm filter; Millipore) as the main carbon source [22].

After incubation, cultures were centrifuged at 3000 rpm for 5 min. Pellets were re-suspended in the same volume of 10 mM $MgSO_4$. Both supernatants and pellets were used to perform BS screening assays: oil displacement assay, drop collapse assay, emulsification

assay and lipase production [23]. All assays were performed in triplicate and sterile Milli-Q water was used as a negative control.

i. Drop collapse assay

Two to three µL of mineral oil were added to each well of a 96-well microtiter plate and allowed to equilibrate 1 h at 37 ºC. Subsequently, 5 µL of culture supernatant or pellet was added to the centre of the wells over the oil film. The shape of the oil drop was examined after 1 min. Flattened drops were considered positive for BS production, and intact drops were considered negative.

ii. Oil displacement assay

Fifteen µL of weathered crude oil were added to a 150 mm-diameter Petri dish containing 40 mL of distilled water. Ten µL of supernatant and pellet suspension were carefully added to the centre of the oil film, and after 30 s of incubation the diameter of the clear halo zone was measured.

iii. Emulsification assay

Four mL of culture supernatant or pellet suspension, and 4 mL of hexadecane or diesel were vortex-mixed for 5 min. The mixture was left undisturbed for 24 h and the height of the emulsion layer was measured. The emulsification activity was expressed as the percentage of the emulsion layer height from the total liquid height.

iv. Lipase production

Agar plates were prepared according to Sriram *et al.* [22]: 2% Tween 80, 2.5% agar, and 0.5% methyl red. Twenty µL of culture supernatant and pellet suspension were added to a cut in the plates, and incubated overnight at room temperature. Strains were considered positive for lipase production when a zone of clearance around the cut was observed.

Biofilm formation assay

This assay is based on the ability of bacterial strains to form biofilms on plastics, usually polystyrene, polypropylene and polyvinylchloride [24–26].

Strains were grown in Luria-Bertani medium (LB) (per L: 10 g tryptone, 10 g NaCl, and 5 g yeast extract) at 30 ºC and 120 rpm for 12-24 h. After incubation, $OD_{600\ nm}$ of the inocula was adjusted to 0.3 using the same LB medium [25]. PS and PP 96-well plates were prepared with a total volume of 300 µL: 3 µL of culture (100-fold dilution), 15 µL of filter-sterilized hydrocarbon (diesel or hexadecane; 5%, v/v) as the main carbon source, and 285 µL of W minimal medium [27] (*n=3*). Negative control wells were prepared with 3 µL of sterile LB medium. The plates were closed with parafilm, covered with aluminium foil, and incubated at 30 ºC for 7 days in static conditions [26].

According to the method described by Tribelli *et al.* [26], after incubation, the supernatant with planktonic cells was very gently pipetted to UV plates to measure absorbance at 600 nm (absorbance of planktonic cells: APL). In the polystyrene or polypropylene plates, 100 µL of 10 mM $MgSO_4$ was added to solubilize the remaining planktonic cells. After approximately 20 min, the $MgSO_4$ was eliminated and 25 µL of 1% crystal violet solution was added (Sigma Aldrich Co, LLC), which stains the biofilm cells

attached to the plastic plates [24]. After 20 min incubation at room temperature, plates were washed 4 times with sterile distilled water, to remove the crystal violet. Attached biofilm cells were solubilized with 200 µL of 96% ethanol and incubated for 20 min. Subsequently, the liquid was transferred to UV-plates and absorbance at 550 nm was measured (absorbance of crystal violet: ACV). Adherence indices were calculated based on both absorbance values, APL and ACV (Equation 1):

$$\text{Adherence index} = \frac{\text{ACV (Absorbance of crystal violet} - \text{Biofilm cells)}}{\text{APL (Absorbance of planktonic cells)}} \qquad \textbf{Equation 1}$$

Screening assay for diesel-degrading capability

The diesel-degrading capability of the bacterial strains was assessed using a protocol modified from Kubota *et al.* [28]. 2,6-dichlorophenol indophenol (DCPIP), an oxidation-reduction indicator, detects the oxidation of NADH to NAD^+ by actively respiring cells. Strains were pre-cultured in rich 869 medium [29] at 30 ºC and 160 rpm until the $OD_{660\ nm}$ was greater than 1. Cultures were centrifuged at 3000 rpm for 5 min, pellets were washed twice with 10 mM $MgSO_4$, and cell density was adjusted to an $OD_{660\ nm}$ of 1 using W medium. Bacterial suspensions (80 µL) were added to 750 µL of sterile W medium (Fe-free), 50 µL of 150 µg mL^{-1} $FeCl_3 \cdot 6H_2O$ solution, 50 µL of 100 µg mL^{-1} 2,6-DCPIP solution and 5 µL of filter-sterilized diesel in 1.5-mL sterile microtubes, and cultivated at 30 ºC and 120 rpm for 48 h. W medium contained (per L): 2.0 g $(NH_4)_2SO_4$, 14.3 g Na_2HPO_4, 5.4 g KH_2PO_4, 0.5 g NaCl, 0.3 g $MgSO_4$, $15.0 \cdot 10^{-3}$ g $CaCl_2 \cdot 2H_2O$, $2.0 \cdot 10^{-3}$ g $ZnSO_4 \cdot 7H_2O$, $1.5 \cdot 10^{-4}$ g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, $2 \cdot 10^{-4}$ g $CuSO_4 \cdot 5H_2O$, $4 \cdot 10^{-4}$ g $CoCl_2 \cdot 6H_2O$, $1.0 \cdot 10^{-3}$ g $MnSO_4 \cdot 5H_2O$, and 0.3 g KNO_3 [27]. After incubation, the colour of the tube was observed,

and appraised as positive for microbial diesel-degradation ability if colourless, and negative if blue. The experiment was repeated with autoclaved cells, to assure that the positive result was due only to biological processes.

Estimation of diesel degradation capacity: in vitro protocol

Three strains (SPG23, PF1 and GK2) were selected for evaluation of their DRO degradation capacity. Strains were pre-cultivated in LB medium for 24 h at 30°C and 150 rpm. Cultures were centrifuged at 3000 rpm for 10 min, and the pellets were washed twice and re-suspended to an $OD_{590\ nm}$ of 1 in Bushnell Haas modified mineral medium (BH2). BH2 medium contained (per L): 1.3 g K_2HPO_4, 1.0 g KH_2PO_4, 0.8 g NH_4Cl, 0.8 g $NaNO_3$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 0.4 g $MgSO_4 \cdot 7H_2O$ [30]. Then, 0.5 mL were added to sterile tubes containing 4.5 mL of BH2 medium and 1 g L^{-1} of filter-sterilized diesel, as the sole carbon source. The tubes were incubated at 30°C and 150 rpm. At each time point (0, 2, 4, 6, 8 and 10 days), six flasks were used for residual hydrocarbon and colony forming units (CFU) determinations. Controls without inoculation were also set up for all time points. A control with heat killed cells (autoclaved inocula) was incubated for 10 days to ensure that hydrocarbon losses were due to bacterial degradation and not to adsorption on cell walls. At each sampling time, 100 µL of serial ten-fold dilutions of the cultures were plated in 1:10 diluted 869 agar medium. After 7 days of incubation at 28 ºC, CFU were determined and calculated per mL of medium.

The diesel hydrocarbons, or diesel range organics (DRO), were extracted from the culture medium by ultrasonic assisted extraction, using an ultrasonic water bath (Ultrasons, J. P. Selecta, S. A.). Closed tubes containing the sample and the extraction solvent, hexane,

at a 1:2 ratio (sample/solvent) were extracted for 1 h. This extraction led to recovery rates of higher than 90% for all DRO analyzed [31]. Then, an aliquot of the organic phase was removed and dried with anhydrous Na_2SO_4. Gas chromatography (Model 450 GC, Agilent Technologies) coupled to a mass spectrometer (Model 220 MS, Agilent Technologies) (GC/MS) was used to analyse the DRO, comprising alkanes from C_{10} (decane) to C_{25} (pentacosane). Before analysis, a mix of deuterated internal standards, containing 1,4-dichlorobenzene-d_4, acenaphthene-d_{10}, chrysene-d_{12}, naphthalene-d_8, perylene-d_{12} and phenanthrene-d_{10} (Internal Standards Mix 33, Dr. Ehrenstorfer) was added to the extracts at 0.2 mg L^{-1}. Calibration of DRO concentrations was carried out with a standard containing a mixture of C_{10}-C_{25} alkanes (DRO mix, Dr. Ehrenstorfer). The calibration standards were prepared in hexane at: 0.1, 0.5, 1.0, 2.5, 5, 7.5 and 10.0 mg L^{-1}. Internal standards were also added to standards in the same concentration as for the samples (0.2 mg L^{-1}). Chromatographic separations were performed on a FactorFour VF-5ms EZ-Guard capillary column (30 m x 0.25 mm x 0.25 µm; Agilent Technologies) that operated with the following oven temperature program: 40 ºC (held for 10 min) to 300 ºC, at 10 ºC min^{-1}. Helium was used as carrier gas, at constant flow of 1 mL min^{-1}. The injector was operated with a temperature ramp from 60 ºC to 300 ºC (held for 35 min), at 200 ºC min^{-1}, and samples (1 µL) were injected in split/splitless mode. The mass spectrometer was operated in full scan mode. Molecules were ionized by electron impact (EI) and the ion trap temperature was set at 220 ºC.

Hydrocarbon degrading kinetics

The diesel degradation kinetic constants of each bacterial strain were calculated using the first order kinetics model (Equation 2), for each alkane (C_{10} to C_{25}) and for the sum of alkanes (∑DRO):

$$ln\frac{C}{C_0} = -k \cdot t$$ **Equation 2**

The rate constant k (day^{-1}) can be obtained from the slope of the linear adjustment of Eq. 2, where C_0 is the initial concentration of the contaminant (mg L^{-1}) and C is the remaining concentration in the liquid medium (mg L^{-1}) at each time, t (days).

Statistical analysis

PASW Statistics software (Version 20.0.0; IBM SPSS Statistics, Inc.) was used to analyze the data. One factor-ANOVA was used to compare the adherence indices in biofilm formation protocol, since Kolmogorov-Smirnov and Levene's tests indicated normally distributed variables with homogeneous variances. A significance level of $p=0.05$ was considered.

RESULTS

Diesel-degrading ability, biosurfactant production and biofilm formation

In order to select strains for detailed analysis, all 19 isolates (Table 1) were evaluated for their ability to metabolize DRO in the presence of a redox indicator, DCPIP. After 48 hours of incubation, all 19 strains scored positive when compared to non-inoculated and autoclaved biomass controls. Given their DRO degrading abilities, all 19 strains were then tested for biosurfactant production and biofilm formation.

The biosurfactant production assays (Table 2) showed positive results for some of the strains tested. In the drop collapse assay, strains SPG23, GK2, PG16, PG24 and PG26 culture supernatant, and the pellet suspensions of strains PF1, PG11 and PG17 showed flattened drops on mineral oil. The culture supernatant of strains PG28, SPG23, GK2, PF1, PG16, PG17 and PG19 produced a clear halo zone, with a diameter of more than 1 cm, in the oil displacement experiment. In general, most of strains were capable of emulsifying hydrocarbons, reflected by a clear emulsified layer. This emulsified layer was developed most clearly in the presence of hexadecane: emulsification activities of the supernatant of strain PG17 and the pellet suspension of strains PG29, SPG23, GK2, PF1, PG10, PG12, PG13, PG16 and PG24 were higher than 50%. Emulsification activity in the presence of diesel was significantly lower: only the emulsification activities of the pellet suspensions of strains PG27, PG10, PG12 and PG17 were equal or higher than 50%. Lipase production was only found with the supernatant of strains PG21, PG27, SPG23, PG8, PG10, PG11 and PG12. In all, strain SPG23 scored positive for all of the traits, and strains GK2, PF1, PG16 and PG17 only got negative results in lipase production test.

For all strains, biofilm adherence indices (Equation 1) in the presence of hexadecane and diesel were higher than the respective non-inoculated control, but only certain strains presented significant differences ($p<0.05$) (Table 3). The strains with the strongest tendency to form biofilms in the presence of hexadecane were PG27, PG28, SPG23, GK1, GK2, PG8, PG19 and PG26, with adherence indices between 17 and 37 times higher than controls in polypropylene plates, and between 18 and 60 times higher than controls in polystyrene plates. With diesel fuel, strains PG27, PG28, SPG23, GK1, GK2 and PG26 in polypropylene

plates had the highest adherence indices, between 18 and 30 times higher than respective controls.

In vitro diesel range organics degradation by selected strains

GC/MS analysis of the sum of the 16 alkanes analyzed (∑DRO, from C_{10} to C_{25}) showed that strains SPG23 and PF1 degraded 27 and 16% of the analyzed ∑DRO, respectively after 10 days (Figure 1). Strain GK2 exhibited more significant DRO removal, at nearly 90% of ∑DRO compared to controls. Low-molecular weight alkanes were removed more efficiently than the high-molecular weight alkanes by all three strains, and removal extents decreased with an increasing number of carbon atoms (Figure 2). The highest degradation capacities of strains SPG23 and PF1 were found for C_{10} and C_{11} (80 - 90% were degraded by strain SPG23, and 75 - 82%, by strain PF1), and the lowest, for C_{13} to C_{25} (17 - 31% were degraded by strain SPG23, and 5 - 15%, by strain PF1). Strain GK2 demonstrated very high degradation efficiencies for all alkanes (higher than 70%), with the sole exception of C_{24} and C_{25}, where approximately 40% was degraded.

The degradation profile of strains SPG23 and PF1 was very similar: the degradation rate was stable from 4-6 days until the end of the experiment, after an acclimatization period of 2 days (Figure 1). Strain GK2 also reached the maximum degradation efficiency at 4-6 days, but on the contrary, it did not show an acclimatization period, and degradation started immediately: 2 days after the start of the experiment it had already degraded more than 40% of ∑DRO present in the medium.

Hydrocarbon removal rates, expressed as *k* values, decreased with increasing carbon number for all strains (Figure 3). Strain PF1 showed the slowest alkane degradation rates, followed by strain SPG23, with strain GK2 demonstrating the fastest removal rates. The degradation rates for C_{10} and C_{11} were similar for all three strains, with the greatest differences being observed for the highest molecular weight alkanes: for C_{12}-C_{25} *k* values varied from 0.044 to 0.114 day^{-1} for strain SPG23; and from 0.05 to 0.042 day^{-1} for strain PF1. Degradation constants (*k*) of strain GK2 were relatively higher than those of SPG23 and PF1 strains (0.117 - 0.344 day^{-1}), reflecting a relatively faster degradation process, except for C_{24} and C_{25} where the lowest *k* values were obtained (0.047 - 0.050 day^{-1}).

DISCUSSION

The success of bioremediation processes is directly related to the metabolic potential of the microorganisms applied [13]. Diesel-degrading bacteria should possess alkane degrading genes, and be characterized by a high degradation efficiency, fast degradation kinetics, and strong substrate affinity. Biosurfactant production and biofilm formation are important properties for the successful application of diesel-degrading bacterial strains in contaminated media: soil is better colonized by biofilm-forming strains, and contaminant bioavailability, which is mostly low in soils due to sorption on organic and mineral soil components, can be significantly increased due to biosurfactant release by the bacterial cells [18,26,32,33].

Most of the bacterial strains isolated presented biosurfactant production according to the results of the protocols performed, although they delivered dissimilar results for some strains (*i.e.* strains PG21, PG29 or PG26 showed a positive outcome for only one assay,

while strains SPG23, GK2, PF1, PG16 and PG17 delivered positive results for 3 or 4 assays performed) (Table 2). Other authors also found similar results while screening biosurfactant production in bioremediation strains. Ibrahim *et al.* [9] screened a variety of strains (*Micrococcus kristinae*, *Bacillus licheniformis*, *Bacillus firmus*, *Bacillus lentus*, *Serratia marcescens*, *Pseudomonas paucimobilis*) isolated from a crude oil contaminated soil, and obtained halos of 23 to 51 mm in an oil displacement assay, positive results for a drop collapse assay, and emulsification activities of 40-90%. This indicated the potential use of those biosurfactant producing strains for soil bioremediation. Sriram *et al.* [23] found a clear halo zone of 2.95 cm^2 in an oil displacement assay, and an emulsification activity with hexadecane of 62% for *Bacillus cereus* NK1, concluding that this strain could be used as a bioremediation tool in crude oil contaminated scenarios. The production of biosurfactants may have improved the DRO degradation performance of the strains tested here. Biosurfactants may provoke the formation of diesel fuel-containing micelles leading to increases in apparent water solubility and decreased interfacial tension. Further, diesel microdroplets may be degraded inside the cells by entering the microbial wall: hydrophilic micelles can be incorporated into the cell membrane, releasing the hydrophobic diesel molecules into the intermembrane space [1,34].

The emulsification activity is one of the most widely used methods to determine the presence of biosurfactants, but the results can significantly vary with the complexity of the hydrocarbons being emulsified. The use of hexadecane and diesel, also revealed that diesel was more difficult to emulsify, as it is a complex mixture of compounds and heavier than hexadecane.

As observed for the biosurfactant production assays, the formation of biofilm was found to be less substantial in the presence of diesel than in the presence of hexadecane (Table 3). Ramey *et al.* [35] reviewed that some species of *Pseudomonas* are known to form biofilms on biotic or abiotic surfaces, which can allow them to establish stable biofilms either on soil or on plant roots. The formation of biofilms might have improved the performance of the strains in the degradation assay. Biofilm forming strains may attach more easily to the diesel substrate than planktonic communities. As cells in a biofilm usually possess a better metabolic capacity and a higher cell density than planktonic cells, this can lead to improved degradation efficiencies.

Strains PF1, SPG23 and GK2 presented a wide range of maximum degradation efficiencies in the *in vitro* diesel degradation test: respectively, 17, 26 and 90% of total DRO was degraded after 10 days (Figure 1). Also other authors reported high diesel degradation capacities by several bacterial strains. Zhang *et al.* [12] also reported a diesel degradation efficiency of 30-60% in culture medium (mineral medium with diesel as sole carbon source) with different surfactants, using *Pseudomonas aeruginosa* endophytes isolated from *Scirpus triqueter*. Deng *et al.* [36] isolated a hydrocarbon-degrading strain, *Achromobacter* sp. HZ01, which degraded up to 90% diesel in 10 days of incubation in minimal salt medium with 2% (*w/v*) of evaporated diesel oil.

PF1, SPG23 and GK2 strains were subjected to whole genome sequencing and genes which are known to be involved in the degradation of alkanes, benzene and naphthalene and biofilm formation were present [20,21], thus confirming their ability for diesel-degradation. However, their degradation performances were very different, indicating that degradation

efficiency cannot be directly related to the presence of the genes. The regulation of gene expression, the involvement of other genes from central cellular metabolism, or the differences in enzyme efficiencies due to dissimilar protein sequences, can involve diverse metabolic pathways and degrading efficiencies in strains possessing hydrocarbon degrading abilities [37]. Other factors, such as the microbial capacity to grow on the specific substrate, biofilm formation or biosurfactant production, can also have a significant influence on degradation performance.

In general, low-molecular weight alkanes were more easily degraded than high-molecular weight alkanes, as they are shorter carbon chains (Figure 2). von der Weid *et al.* [11] also reported higher degradation rates of the lightest alkanes with *Dietzia cinnamea*. Hydrocarbon degradation also slowed down as the complexity of the contaminant molecules increased (Figure 3). This was especially significant for strain PF1, since the kinetic constants substantially decreased for alkanes with more than 12 carbons. In contrast, strain GK2 presented very high degradation efficiency for all alkanes, with the sole exception of C_{24} and C_{25}. Strain GK2 was also the fastest degrader of the strains used, and the degradation rate only decreased significantly for alkanes of 23 carbons or more, as found for the degradation efficiency. This indicated that strain GK2 could degrade more complex mixtures of hydrocarbons with a higher efficiency than strains SPG23 and PF1, which only degraded the lightest compounds (C_{10} and C_{11}) at a high rate.

Strain PF1 demonstrated the poorest degradation results, and also low adherence indices in biofilm production assays. Strain SPG23 showed a slightly better degradation performance than PF1, biofilm formation, and lipase production, which strains PF1 and

GK2 did not show. Strain GK2 showed the best degradation efficiencies and rates for all alkanes, with high biofilm adherence indices and biosurfactant production confirmed by all assays (except lipase). Microbial biosurfactants might have enhanced the performance of this strain (by increasing diesel bioavailability), but it seems that the biofilm formation capacity might have had a more important role in the high *in vitro* degradation efficiency of strain GK2, probably due to a better attachment to diesel.

Therefore, our results indicate that strain GK2 is a promising candidate to be used in bio- and/or phyto- remediation trials with diesel-contaminated soils. This strain was previously isolated from a diesel-contaminated site (from rhizospheric soil) were poplars were used for plume hydraulic controlling, and may be easily adapted to be used for remediation of this particular contaminated site, as an indigenous bioaugmentation inoculant in combination with poplars, or in other contaminated soils. Besides, the presence of plant growth-promoting genes spread throughout its genome (Panagiotis Gkorezis, personal communication, February 2017) corroborates once more the potential of this strain to be used in association with plants in phytoremediation. Indeed, the performance of this strain has been recently tested in a rhizodegradation pot experiment using diesel-contaminated soil, and it significantly increased the degradation of diesel, specially of the longest alkanes (from C_{19}) [38], which is consistent with the results of this work.

CONCLUSIONS

Bioremediation efficiency will highly depend on several microbial features, including biosurfactant production, biofilm formation, and the presence and expression of hydrocarbon degrading genes. Strain GK2 showed very good results, degrading, in general,

90% of all the alkanes present, including the higher molecular weight DRO. This is an important feature for a diesel-degrading strain, since in soil remediation it is essential to efficiently eliminate all compounds present in such a complex mixture of different molecules as diesel. Most likely, biosurfactant production improved the degradation performance of strain GK2 for the longest alkanes, compared to the other strains; also the high adherence indices might have been essential, due to a better attachment to diesel droplets.

The selected strains (SPG23 (*Arthobacter* sp.); PF1 (*Acinetobacter oleivorans*); and GK2 (*Acinetobacter calcoaceticus*)) presented alkane-degrading genes spread in the genome, but significant differences were found in their degrading efficiencies and kinetics. This indicated the need for further study in gene expression coding for metabolic pathways in order to more adequately select the most efficient strains to be applied in bioremediation scenarios.

Further investigations are also required with respect to the characterization of the biosurfactants produced, the formation of biofilms in soil media, and the microbial adherence to hydrocarbons, as well as determining the optimum conditions for the successful application of these bacterial strains in soil remediation.

ACKNOWLEDGEMENTS

This research was supported by ERDF Interreg Sudoe Program (PhytoSUDOE-SOE1/P5/E0189); by the Galician government (Agrupación Estratéxica CRETUS;

AGRU2015/02); and by the University of Hasselt (BOF project 06G02 and Methusalem project 08M03VGRJ).

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Table 1. Collection of bacterial strains isolated from a contaminated site and selected for this study.

Phylum	**Species**	**Strain**	**GenBank***
Actinobacteria	*Arthrobacter* sp.	PG21	KU350608
	Brevibacterium sp.	PG27	KU350614
	Microbacterium sp.	PG28	KU350615
	Microbacterium hydrocarbonoxydans	PG29	KU350616
	Arthrobacter sp.	SPG23	KU350590
Proteobacteria	*Acinetobacter calcoaceticus.*	GK1	KU350617
	Acinetobacter calcoaceticus	GK2	LQMV01000001
	Acinetobacter oleivorans	PF1	KU350618
	Acinetobacter calcoaceticus	PG7	KU350598
	Acinetobacter calcoaceticus	PG8	KU350599
	Pseudomonas reinekei	PG10	KU350591
	Pseudomonas putida	PG11	KU350601
	Pseudomonas sp.	PG12	KU350602
	Pseudomonas koreensis	PG13	KU350603
	Pseudomonas brassicacearum	PG16	KU350593
	Pseudomonas brassicacearum	PG17	KU350605
	Acinetobacter calcoaceticus	PG19	KU350619
	Pseudomonas sp.	PG24	KU350611
	Acinetobacter sp.	PG26	KU350613

**NCBI GenBank accession number: 16S ribosomal RNA gene partial sequence*

Table 2. Results of the biosurfactant production screening assays using supranatant (SN) and pellet suspensions (PEL) for each strain.

Strain	Drop collapse[1]		Oil displacement (halo diameter, cm)		Emulsification assay (emulsification activity, %)[2]				Lipase production	
					Hexadecane		Diesel			
	SN	PEL	SN	PEL	SN	PEL	SN	PEL	SN	PEL
PG21	-	-	0.5	0.5	50	SL	SL	30	+	-
PG27	-	-	0.3	0.3	30	40	-	50	+	-
PG28	-	-	1.4	-	-	10	-	40	-	-
PG29	-	-	0.4	-	-	60	15	40	-	-
SPG23	++	-	1.1	0.4	40	60	SL	SL	+	-
GK1	-	-	0.6	-	-	50	-	25	-	-
GK2	++	-	1.3	-	-	60	SL	SL	-	-
PF1	-	+	1.2	-	-	60	SL	SL	-	-
PG7	-	-	0.5	-	-	25	SL	30	-	-
PG8	-	-	0.9	-	-	50	-	20	+	-
PG10	-	-	0.8	0.3	30	60	SL	55	+	-
PG11	-	++	-	-	-	40	SL	35	+	-
PG12	-	-	1	0.5	50	60	-	50	+	-
PG13	-	-	0.6	0.4	40	60	5	45	-	-
PG16	++	-	1.1	0.5	50	65	10	40	-	-
PG17	-	+	1.2	0.6	60	-	-	50	-	-
PG19	-	-	1.3	-	-	50	-	25	-	-
PG24	+	-	0.4	0.4	40	60	SL	35	-	-
PG26	++	-	0.5	0.4	40	40	-	15	-	-

Negative results in all protocols are indicated with "-".

[1]Positive results were comparatively characterized as + and ++ according to the degree of drop flattening.

[2]Strains presenting only a slight emulsification layer are detailed as "SL" (slight layer).

Table 3. Adherence indices (obtained from Eq. 1) of the bacterial strains in polypropylene (PP) and polystyrene (PS) plates in the presence of hexadecane or diesel. The results are expressed as the mean ± the standard error (*n=3*). Significant differences with the respective control are indicated with an asterisk (*p<0.05)*.

	Hexadecane						**Diesel**					
Strain	**PP**			**PS**			**PP**			**PS**		
Control	3.5	±	0.4	1.9	±	0.6	3.4	±	0.4	2.3	±	0.2
PG21	10.1	±	0.5	10.6	±	4.7	6.0	±	2.7	8.5	±	1.2
PG27	81.0	±	5.1*	38.4	±	3.3*	2.2	±	0.7	53.4	±	10.5*
PG28	61.7	±	8.2*	68.4	±	2.7*	3.2	±	1.3	68.0	±	2.3*
PG29	35.5	±	9.5	12.6	±	2.1	4.1	±	0.8	6.6	±	0.3
SPG23	62.6	±	2.2*	55.5	±	2.9*	5.8	±	1.4	43.1	±	2.3*
GK1	80.6	±	12.5*	39.1	±	9.8*	3.1	±	0.1	42.2	±	2.1*
GK2	128.1	±	2.6*	114.0	±	6.0*	6.1	±	2.0	46.0	±	6.2*
PF1	16.7	±	2.1	15.0	±	1.8	3.1	±	0.8	13.3	±	2.3
PG7	10.0	±	2.0	8.6	±	0.5	3.2	±	1.1	10.3	±	0.4
PG8	75.1	±	8.7*	47.9	±	3.1*	1.4	±	0.4	26.6	±	2.6
PG10	20.6	±	0.6	16.0	±	3.6	6.1	±	1.2	16.3	±	1.7
PG11	4.2	±	0.8	7.3	±	0.2	4.3	±	2.2	7.3	±	0.8
PG12	30.8	±	7.5	22.7	±	3.2	2.2	±	0.8	12.1	±	1.3
PG13	35.5	±	5.3	22.6	±	1.8	3.7	±	0.1	19.2	±	3.4
PG16	14.1	±	2.4	10.6	±	1.9	5.2	±	1.6	11.8	±	1.8
PG17	9.1	±	1.7	19.4	±	3.1	3.2	±	0.5	12.1	±	4.4
PG19	71.7	±	12.9*	51.0	±	5.1*	4.1	±	1.4	12.6	±	0.2
PG24	23.9	±	3.9	14.6	±	0.9	2.0	±	0.8	22.2	±	11.6
PG26	74.8	±	7.0*	51.1	±	1.1*	2.7	±	0.1	62.4	±	5.3*