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Biofilm lifestyle enhances diesel bioremediation and biosurfactant production in the Antarctic polyhydroxyalkanoate producer *Pseudomonas extremaustralis*

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Abstract Diesel is a widely distributed pollutant. Bioremediation of this kind of compounds requires the use of microorganisms able to survive and adapt to contaminated environments. *Pseudomonas extremaustralis* is an Antarctic bacterium with a remarkable survival capability associated to polyhydroxyalkanoates (PHAs) production. This strain was used to investigate the effect of cell growth conditions—in biofilm versus shaken flask cultures—as well as the inocula characteristics associated with PHAs accumulation, on diesel degradation. Biofilms showed increased cell growth, biosurfactant production and diesel degradation compared with that obtained in shaken flask cultures. PHA accumulation decreased biofilm cell attachment and enhanced biosurfactant production. Degradation of long-chain and branched alkanes was observed in biofilms, while in shaken flasks only medium-chain length alkanes were degraded. This work shows that the PHA accumulating bacterium *P. extremaustralis* can be a good candidate to be used as hydrocarbon bioremediation agent, especially in extreme environments.

Keywords Biofilm · Diesel bioremediation · Biosurfactant production · Polyhydroxyalkanoates · *Pseudomonas*

Introduction

Environmental damage due to oil and its derivatives has become a tough problem worldwide. One of the most widely distributed of these compounds is diesel, a complex mixture of *n*-alkanes, branched alkanes, and small amounts of aromatic moieties.

Bioaugmentation strategies designed to remediate oil contaminated sites should consider that the introduced bacteria must be able to adapt to both abiotic and biotic stresses present in those environments (van Veen et al. 1997; Tyagi et al. 2011). Thus, the presence of some intrinsic mechanisms to cope with those stresses is a desirable characteristic.

Biofilm formation represents a protected way of life that allows cells to survive in hostile environments, to cope with the stress and also to disperse in order to colonize new niches (Decho 2000). Biofilms are described as an efficient biological tool for oil and grease removal due to the high microbial biomass yield and the ability to immobilize compounds (El-Masry et al. 2004; Singh et al. 2006).

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Synthesis of biopolymers like polyhydroxyalkanoates (PHAs) enhances the capability to deal with environmental stress in many bacterial strains. PHAs are reduced carbon storage compounds accumulated by a great number of bacterial species during unbalanced growth conditions, endowing bacteria with enhanced survival, competition abilities, and stress tolerance, and increasing fitness in changing environments (López et al. 1995; Ruiz et al. 2004; Pham et al. 2004; Kadouri et al. 2005; Ayub et al. 2009), including hydrocarbon contaminated environments. Most of the work regarding to PHA and bioremediation are focused on the conversion of toxic petrochemical wastes into valuable biopolymers (Ni et al. 2010), but the advantages conferred by polymer accumulation's capability in the adaptability to oil contaminated environments has not been studied in detail. To our knowledge there is currently no information about how biofilm formation and PHA accumulation affect bioremediation.

One of the main reasons for the persistence of hydrocarbons in the environment is their low bioavailability due to their hydrophobic nature. A possible way to enhance their bioavailability and, thereby, their biodegradation is the application of (bio) surfactants. Many bacterial species are capable of synthesizing these compounds (Ron and Rosenberg 2002) which in turn affect biofilm structures, allowing cell dispersion and colonization (Pamp and Tolker-Nielsen 2007). Surfactant and PHA metabolisms seem to be related because both involve 3-hydroxyacids as intermediate compounds in their biosynthesis (Soberón-Chávez et al. 2005).

Pseudomonas extremaustralis is a novel bacterial species isolated from an Antarctic environment that is able to synthesize polyhydroxybutyrate (PHB), a type of PHA (López et al. 2009). This strain, isolated from a pristine environment, was selected for this study because of its capability to grow in extreme environments as the Antarctica, its stress resistance to low and high temperatures (Ayub et al. 2004, 2009), its high rate of PHA accumulation, and its tendency to form biofilms in stressful environments.

These characteristics led us to hypothesize that *P. extremaustralis* could be able to tolerate the stressful conditions encountered in hydrocarbon contaminated sites, making it a candidate for bioremediation purposes. The aim of this work was to analyze if biofilm formation and PHA accumulation in *P. extremaustralis* affect diesel remediation.

Materials and methods

Bacteria and growth conditions

Pseudomonas extremaustralis DSM 17835 or a recombinant strain carrying plasmid pGEc47 (Eggink et al. 1987), *P. extremaustralis*/pGEc47, were grown under PHA accumulating conditions using modified E medium (Vogel and Bonner 1956) supplemented with 1% MT microelements (Lageveen et al. 1988), 1 mM $MgSO_4 \cdot 7\ H_2O$, 0.25% w/v sodium octanoate (Sigma) and 0.08% KNO_3. For non-PHA accumulating growth conditions, Luria–Bertani medium (LB) was used and tetracycline 10 µg/ml was added to maintain plasmid pGEc47. Overnight cultures grown in conditions favoring or not favoring PHA accumulation were used to inoculate E2 minimal medium (Lageveen et al. 1988) supplemented with 5% diesel (Petrobras) at an initial OD_{600} of 0.025 or 0.05 for biofilm and shaken flask assays, respectively. Controls without carbon source were also run. Biofilm cultures were conducted on polystyrene microtiter plates (Gibco), as described by O'Toole and Kolter (1998). Briefly, 200 µl of each culture were added to the microplate wells and developed for 7 days at 30°C without agitation, in a wet chamber to prevent evaporation. Shaken flask assays were carried out in 500 ml capped bottles containing 25 ml of culture. Cultures were incubated for 7 days at 30°C and 300 rpm. Growth in biofilms and shaken flasks was monitored by measuring OD_{600}. In the case of biofilms, total bacteria, including surface attached and planktonic cells from eight wells, were resuspended and OD_{600} was measured. PHA accumulation was checked using Nile Blue stain (Ostle and Holt 1982).

Biofilm formation analysis

After 7 days of biofilm development, non-attached cells were collected and OD_{600} was measured (absorbance of planktonic cells: APL). Biofilm attached cells were stained with crystal violet (O'Toole and Kolter 1998). Briefly, attached cells were dyed with 200 µl 0.1% crystal violet. After 20 min, the unbound crystal violet solution was removed and plates were gently rinsed with water. Subsequently, the dye was extracted in 200 µl 96% ethanol for 20 min and transferred to flat bottom microtiter plates in order to measure the

absorbance at 550 nm (absorbance of crystal violet: ACV) in a Tasoh Corp MPR A4i microplate reader. The adherence index was defined as ACV/APL.

Detection of alkane monooxygenase gene alkB

PCR amplification was done using the degenerated primers alkB-1f and alkB-1r designed by Kloos et al. (2006). The obtained fragment was sequenced (Macrogen Inc., Korea), the sequence was analyzed using BLAST tools (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al. 1997) and deposited in the EMBL Nucleotide Sequence Database under Accession No. FR745429. The phylogenetic tree was constructed using the neighbor joining method of Saitou and Nei (1987), included in MEGA3 package (Kumar et al. 2004). The tree topology was tested by bootstrap analysis of 1,000 resamplings.

Diesel biodegradation

Eight biofilm wells or one capped bottle were sacrificed as experimental units after 7 days of cultivation. For each assay, 3 experimental units with their respective controls without bacteria were used. Remnant diesel was measured using modified EPA 8015M technique (US EPA 2001). Briefly, one complete experimental unit was acidified to pH = 2 and then extracted with 20% v/v *n*-hexane. 2-Fluorobiphenyl was used as internal standard. The solvent phase was collected and dehydrated by eluting it through a Pasteur pipette filled with anhydrous sodium sulphate. The samples were analyzed by GC/MS using a Shimadzu GC-17A GC gas chromatograph coupled with a Shimadzu GCMS-QP5050A mass spectrometer. The column used was a Tracsil TBR-1 (60 m by 0.32 mm) with Nitrogen as carrier gas at a flow rate of 0.7 ml min^{-1}. The injector temperature was 250°C, and the detector's 280°C. All samples were run at 60°C for 10 min, then ramped to 155°C at 5°C min^{-1}, 1 min at 155°C then to 250°C at 10°C min^{-1} 148 and then held for 50 min. The percentage of residual diesel was calculated comparing the area of the GC peaks of the cultures with those of the control without bacteria.

Biosurfactant determination

A pool of 30 wells of biofilm or 5 ml of shaking flask cultures were collected and the aqueous phase was centrifuged for 10 min at 4,000 rpm to obtain the cell-free supernatant. Biosurfactant production was estimated by measuring surface tension with a Du-Nouy tensiometer.

Statistical analysis

Differences were analyzed using two-tails *t* tests performed with GraphPad software.

Results

Cell growth with 5% diesel as sole carbon source

Two different conditions, static biofilms and agitated flasks, were used to determine the growth capability of *P. extremaustralis* using diesel as sole carbon source. Strong differences in the growth of the cells, measured as OD_{600}, were observed. While in biofilms the total OD_{600} increased 15-fold, the cultures growing in shaken flasks only increased 1.5-fold (Fig. 1). The presence of PHA in the inoculum always showed a slight but not significant growth increase in both conditions. No growth was observed in controls without diesel (Fig. 1). On the other hand, no polymer was detected by Nile blue staining after 24 h of cultivation with diesel as carbon source (data not shown); indicating that the prior accumulated PHA

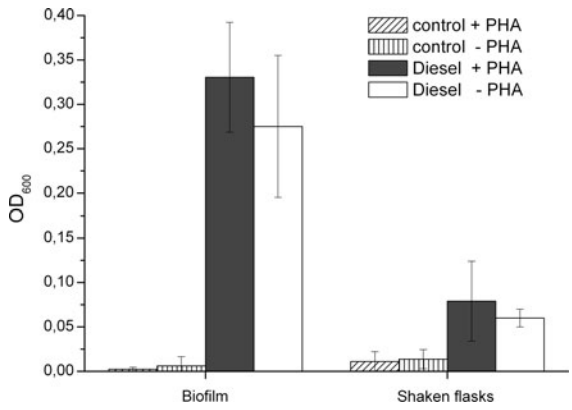

Fig. 1 Growth of *P. extremaustralis* in biofilm and shaken flasks containing 5% diesel after 7 days. *Bars* represent mean ± standard deviation of triplicates experiments. *Control* + or −*PHA* inocula that had accumulated (+) or had not accumulated (−) PHA, growing without diesel. *Diesel* + or −*PHA* inocula that had accumulated (+) or had not accumulated (−) PHA, growing with diesel as carbon source

was metabolized. The analysis of the biofilm adherence index (ACV/APL) showed significant differences ($p = 0.044$) reaching values of 1.95 ± 0.495 and 3.48 ± 0.77, when using inocula containing PHA compared to non-PHA containing inocula. This suggests that PHA conferred an adaptive advantage to the cells, enabling them to grow unattached to surfaces, where diesel is more available.

Screening of alk genes

The capability of growing in diesel by degrading *n*-alkanes (the mayor constituent of diesel oil) is associated to the presence of *alkB*. Using *alkB* degenerated primers, we searched for this gene in the genome of *P. extremaustralis*. We obtained a 392 bp amplification fragment that showed over 80% nucleotide identity with *alkB* (coding for alkane hydroxylase) from several *Pseudomonas* isolates. The highest nucleotide identity (96%) corresponded to a putative *alkB* of *Pseudomonas* sp. PT03 (Accession No: DQ182286.1).

In order to verify if the presence of extra copies of the *alkB* gene could support growth in shaken flask conditions, *P. extremaustralis*/pGEc47, a recombinant strain bearing the gene belonging to *P. putida* GPo1, was assayed. This recombinant strain was able to grow in shaken flasks reaching 4.1-fold of the initial OD. No differences were observed between *P. extremaustralis* and *P. extremaustralis*/pGEc47 when grown in biofilm conditions. A comparison between the amino acid sequences of AlkB belonging to *P. extremaustralis* and *P. putida* GPo1 resulted in only 41% identity. A phylogenetic analysis showed a clear similarity of AlkB of *P. extremaustralis* with other long chain alkane monooxygenases, i.e. *n*-alkane 1 monooxygenases of *Pseudomonas fluorescens* CHA0's and *Rhodococcus* sp. Q15, resulting in an amino acid similarity of 82 and 63%, respectively (Fig. 2).

Diesel degradation

In order to investigate if diesel degradation is affected by cell growth conditions and/or the presence of PHA in the inoculum, diesel content was measured at the end of the experiment in both inoculated and non inoculated control cultures. After 7 days, the residual diesel chromatograms showed no differences between cultures that had accumulated or had not accumulated PHA (Supplemental Material 1). Due to the nature of our experiments, further evaporation of the small-volatile alkanes was observed. Because of this, the smallest alkane resolved in biofilms was decane, while in the shaken flasks it was octane (Supplemental Material 1 and 2).

Under biofilm lifestyle, total diesel degradation reached 20% compared with a non inoculated control, whereas in the shaken flasks, degradation barely reached 5% (Fig. 3). Diesel degradation in the biofilms affected long-chain alkanes (C14 and up) including recalcitrant branched alkanes like pristane with a degradation of $27.3 \pm 6.0\%$ (Fig. 3). A similar

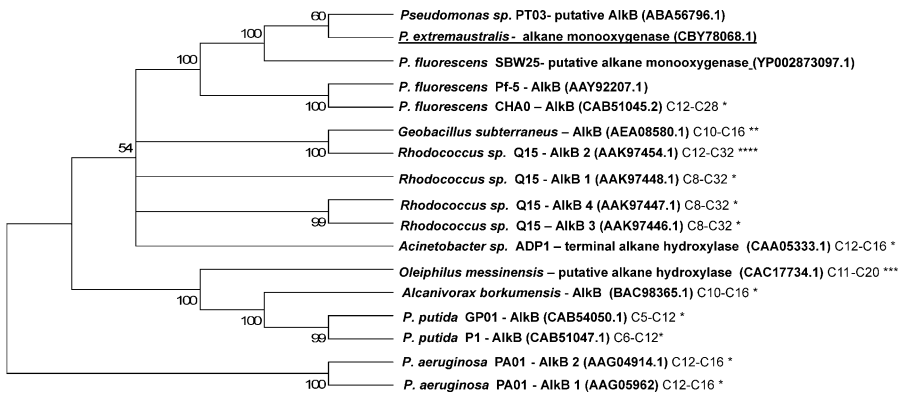

Fig. 2 Phylogenetic tree of *P. extremaustralis* AlkB and related species by the neighbor joining method. Bootstrap values (%) are indicated at the *nodes*, accession number for each microorganism is shown in *parentheses* after the species name. The hydrocarbon chain length degraded by each strain is shown after accession number. *Van Beilen et al. (2003), ** Logan et al. (2009), *** Golyshin et al. (2002) **** Whyte et al. (2002)

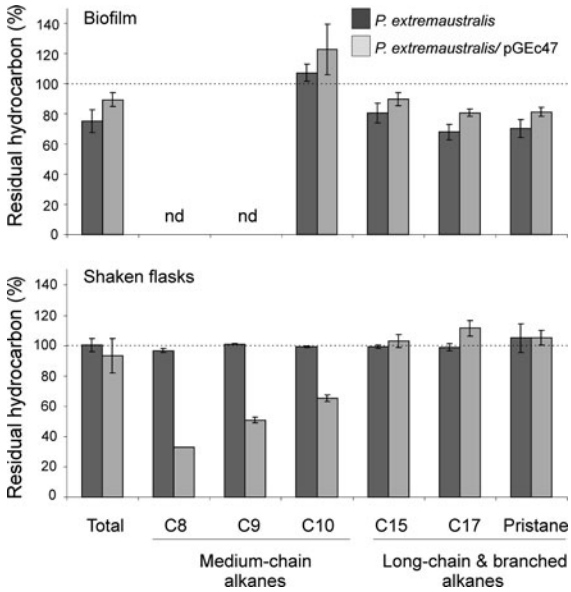

Fig. 3 Comparison of alkanes utilization by *P. extremaustralis* and *P. extremaustralis/pGEc47* growing in biofilm or shaken flask cultures. Control without inocula was considered as 100% (*dotted line*). *Bars* represent mean ± standard deviation of triplicates experiments

degradation, 29.7 ± 10.0%, was observed with phytane (Supplemental Material 1a). A non significant degradation was obtained in the shaken flasks (Fig. 3).

The recombinant strain *P. extremaustralis*/pGEc47, showed a significant degradation of *n*-octane, *n*-nonane and *n*-decane. No significant degradation of the long chain alkanes was observed. Under biofilm growth conditions, *P. extremaustralis*/pGEc47 showed a degradation pattern similar to that of the wild type strain (Fig. 3).

Surfactant production

To analyze if *P. extremaustralis* was able to synthesize biosurfactants, the change in the surface tension was assayed in cell-free supernatants. Shaken flask cultures showed no differences in the surface tension in comparison with the control (Table 1). By contrast, a significant reduction in the surface tension was observed in biofilm cultures when compared with the control without bacteria ($p = 0.001$). PHA accumulation in the inoculum of biofilm cultures stimulated biosurfactant production causing a significant decrease in surface tension supernatants ($p = 0.029$) compared to biofilm cultures inoculated with cells with no prior PHA accumulation (Table 1).

Table 1 Surface tension in dyn/cm of cell free supernatant from inoculum that had accumulated PHA (+PHA) or had not accumulated PHA (−PHA) compared with a control without bacteria (control)

	Surface tension (dyn/cm)	
	Biofilm	Shaken flask
Control	67.67 ± 2.60	67.80 ± 1.64
+PHA	58.90 ± 0.54	65.30 ± 2.95
−PHA	61.37 ± 0.90	64.73 ± 2.13

Discussion

Pseudomonas extremaustralis showed a differential growth in biofilm and shaken flask cultures when diesel was the sole carbon source. In biofilms, *P. extremaustralis* was able to grow using long chain alkanes. These results are consistent with the high similarity found between AlkB of *P. extremaustralis* and AlkB of *P. fluorescens* CHA0 and *Rhodococcus* sp. Q15, which are able to degrade only alkanes longer than C12 (van Beilen et al. 2003; Whyte et al. 2002). In shaken flasks, *P. extremaustralis* barely increased its biomass, but when *alkB* of *P. putida* GPo1 inserted in the medium number copy plasmid pGEc47 was incorporated, it was able to grow. The product of *P. putida* GPo1 *alkB* is a short chain alkane monooxygenase (C5–C12) (Rojo 2005). Only alkanes shorter than C12 were degraded by *P. extremaustralis*/pGEc47 in the shaken flask cultures. No long chain alkanes were degraded in this condition. On the contrary, the degradation capability of *P. extremaustralis*/pGEc47 when growing in biofilms was similar to the wild type strain, as it was only able to degrade long chain and branched alkanes.

Differences in the length of the alkanes degraded in biofilms and in shaken flask cultures can be attributed to AlkB. A possible explanation would be that this protein could be preferentially expressed at high cell densities or have a higher affinity for oxygen, making it more active in biofilms. It has been described that *P. aeruginosa* PAO1's AlkB2 is expressed in early exponential phase and repressed when growth rate decreases, while AlkB1 is expressed at high cell

densities and has a higher affinity for oxygen (Marín et al. 2003). It has also been reported that several organic pollutants have toxic effects on the microbial communities by triggering oxidative stress (Shao et al. 2010). It is probable that c conditions in the biofilm allowed the bacteria to cope with the oxidative stress caused by the presence of the pollutant. In hydrocarbon contaminated microaerobic environments, oxygen could be mostly used for hydrocarbon degradation and less used as electron acceptor for respiration, if other molecules such as NO_3^- or SO_4^{2-} are present (Chayabutra and Ju 2000).

Several reports show that a stressful environment enhances cell attachment (Costerton et al. 2003, Pumbwe et al. 2007) thus; a high adherence index could indicate that in this condition, cells are more stressed than those that present a lower adherence index. The lower adherence in PHA accumulating conditions could suggest that the polymer conferred an advantage in the stressful environment caused by the presence of hydrocarbons. These results are in accordance with those obtained in cold stress assays, where *P. extremaustralis* with accumulated PHA were able to release from the biofilm and acquire a planktonic lifestyle (Tribelli and Lopez 2011).

Most alkane-degrading bacteria are able to secrete biosurfactants to facilitate hydrocarbon accessibility (Ron and Rosenberg 2002). Our results showed that the presence of PHA in the inocula increased surfactant production. PHAs degradation release 3-hydroxyacyl-CoA that could be used for biosurfactant production (Soberón-Chávez et al. 2005). In this case, the presence of PHA could not only enhance stress resistance but also provide precursors for the synthesis of a relevant compound involved in hydrocarbon degradation. Biosurfactant production could affect early steps of biofilm formation, probably leading to a better balance between planktonic and sessile cells in the biofilm.

Successful application of bioaugmentation techniques depends not only on the isolation of the most vigorous degrader strain but also on the selection of an adaptable one (Thompson et al. 2005). The use of microorganisms that accumulate PHAs such as *P. extremaustralis* to inoculate diesel contaminated sites could be a good bioaugmentation strategy, as the capacity to accumulate PHAs increases biosurfactant production and bacterial adaptation. In this work we have demonstrated that *P. extremaustralis* biofilm formation and PHA accumulation capabilities enhance its potential as a bioremediation tool by promoting cell growth, biosurfactant production and long chain and branched alkanes degradation.

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