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Microaerophilic alkane degradation in *Pseudomonas extremaustralis*: a transcriptomic and physiological approach

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Abstract Diesel fuel is one of the most important sources of hydrocarbon contamination worldwide. Its composition consists of a complex mixture of *n*-alkanes, branched alkanes and aromatic compounds. Hydrocarbon degradation in *Pseudomonas* species has been mostly studied under aerobic conditions; however, a dynamic spectrum of oxygen availability can be found in the environment. *Pseudomonas extremaustralis*, an Antarctic bacterium isolated from a pristine environment, is able to degrade diesel fuel and presents a wide microaerophilic metabolism. In this work RNA-deep sequence experiments were analyzed comparing the expression profile in aerobic and microaerophilic cultures. Interestingly, genes involved in alkane degradation, including *alkB*, were over-expressed in micro-aerobiosis in absence of hydrocarbon compounds. In minimal media supplemented with diesel fuel, *n*-alkanes degradation (C13–C19) after 7 days was observed under low oxygen conditions but not in aerobiosis. In-silico analysis of the *alkB* promoter zone showed a putative binding sequence for the anaerobic global regulator, Anr. Our results indicate that some diesel fuel components can be utilized as sole carbon source under microaerophilic conditions for cell maintenance or slow growth in a *Pseudomonas* species and this metabolism could represent an adaptive advantage in polluted environments.

Keywords *Pseudomonas extremaustralis* · Micro-aerobiosis · *alkB* · RNA-seq · Alkane degradation

Introduction

Bacterial adaptability to different environmental conditions requires different strategies, including the response of individual genes or operons and complex regulatory networks that coordinate the control of several genes [25]. Oxygen availability is a key factor in bacterial physiology; in the environment, the uneven distribution of water flow, nutrients, and microbial populations creates a dynamic spectrum of aerobic, microaerophilic, and anaerobic conditions [9]. The heterogeneity of oxygen distribution requires physiological responses that could include the capability to use different electron acceptors as well as the fermentation of carbon compounds [32].

Hydrocarbon contamination has become a tough problem worldwide. One of the most widely distributed sources of this kind of contamination is diesel fuel, a complex mixture of *n*-alkanes, branched alkanes and aromatic hydrocarbons. Due to the lack of functional groups and low water solubility, aliphatic hydrocarbons exhibit both low chemical reactivity and bioavailability for microorganism [7]. *Pseudomonas* species are capable of using *n*-alkanes as carbon source by activating the hydrocarbon as a key first step [5]. This reaction is mostly driven by the enzyme 1-alkane

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monooxygenase encoded by *alkB* [11]. It has been described that degradation of *n*-alkanes is initiated by the oxidation of a terminal methyl group to render a primary alcohol, which gets further oxidized to an aldehyde, and finally converted into a fatty acid [30], which is finally processed by β-oxidation to generate acetyl-CoA [42]. This process has been described to occur under aerobic conditions especially in *Pseudomonas* species [31].

Pseudomonas extremaustralis is a bacterium isolated from Antarctica that shows high stress resistance in association with the production of high amounts of polyhydroxyalkanoates [23]. This strain is capable of growing and developing biofilms under low temperatures and survives freezing [3]. Additionally, the microaerophilic metabolism of *P. extremaustralis* has been studied and the role of the anaerobic global regulator, Anr, has been analyzed in polyhydroxybutyrate metabolism, redox state, oxidative stress resistance under low oxygen conditions and biofilm development [35, 37, 38]. It was also described that *P. extremaustralis* was able to grow using diesel fuel as sole carbon source only when cultured in biofilm [36].

RNA-deep sequencing is a powerful tool for analyzing gene expression and has been used to study the transcriptome profile under different conditions, for example, in *P. aeruginosa* PAO-1 under stress conditions, and *P. putida* KT2440 and *P. extremaustralis* under low temperature conditions [14, 15, 39].

In this work the alkane degradation pathway of *P. extremaustralis* at low oxygen tensions using both high throughput experiments and physiological assays in planktonic cultures was explored. The results demonstrate that 1-alkane monooxygenase is over-expressed under low oxygen conditions regardless of the carbon source, in line with the degradation of alkanes that occurs mainly under low oxygen conditions. This alkane degradation metabolism allows supporting bacterial survival in a newly contaminated environment where these slow growing microorganisms could be the first step in microbial succession. The obtained results provide new information that could be important to improve bioremediation strategies by the stimulation of these microorganisms when oxygen is a limiting factor as found after a recent oil spill.

Materials and methods

Strains and cultured conditions

Pseudomonas extremaustralis—a bacterium isolated from a temporary pond in Antarctica [23] was used through the experiments. Experiments under microaerophilic condition were carried out in hermetically sealed bottles using a 1:2 medium-to-flask volume ratio without shaking, while aerobic condition experiments were performed in shaken Erlenmeyers using 1:10 medium-to-flask volume ratio at 200 RPM. For RNA-seq experiments cultures were grown in LB medium supplemented with KNO_3 (0.08%) at 30 °C. In hydrocarbon degradation experiments pre-inocula were grown overnight in LB medium supplemented with 0.25% sodium octanoate. These cultures were used to inoculate sealed bottles in E2 minimal medium [19] supplemented with 1% diesel fuel (YPF—Yacimientos Petroliferos Fiscales-Argentina) at an initial OD_{600nm} of 0.05. When necessary, 0.05% glucose and/or 0.08% KNO_3 were added. Cultures were incubated at 30 °C under microaerophilic conditions for 7 or 30 days and under aerobic conditions for 7 days. For colony forming units (CFU) assay, serial dilutions of cultures growing with or without diesel fuel as sole carbon source were plated in LB agar until colony development.

RNA extraction and RNA library preparation

Total RNA was isolated from *P. extremaustralis* cultures using Trizol method as described by Gomez-Lozano [15]. Cultures under aerobic or microaerophilic conditions were harvested after 24 h for RNA extraction. RNA quality was analyzed using an Agilent Bioanalyzer. rRNA depletion was performed using the MICROBExpress Kit (Ambion) with the addition of 5S oligonucleotides as was previously described [15]. The samples were validated with an Agilent 2100 Bioanalyzer (Agilent Technologies) after each step, and the final concentration was measured using a Qubit 2.0 Fluorometer (Invitrogen). Directional libraries were prepared with ScriptSeq v2RNA-Seq Library Preparation Kit (Epicentre) and were sequenced using the Illumina HiSeq 2000 platform with a paired-end protocol. Generated reads were of 100 nt lengths. For each condition duplicated independent RNA extraction and libraries were used.

RNA-seq data analysis

Data were de-multiplexed by Beckman Coulter Genomics. Reads alignment in *P. extremaustralis* genome and transcript abundance levels were quantified using the reads per kilobase per million mapped reads (RPKM) performed with the Rockhopper software [27] using default parameters. Differential gene expression was considered only with $P < 0.05$ and $Q < 0.05$. Spearman correlation analysis of normalized counts was performed to verify the concordance between replicates in aerobic and microaerophilic cultures (Fig. S1). Functional enrichment of differentially expressed genes was determined using Blast2GO software [10] by assigning the GO category to all genome sequences and to the differentially expressed genes. Additionally, in silico metabolic analysis was performed using Ipath2 software [43].

Quantitative real time PCR experiments (RT qPCR)

Total RNA of *P. extremaustralis* was extracted from E2 cultures supplemented with diesel fuel, diesel fuel + KNO_3 or LB cultures supplemented with KNO_3 under both aerobic and microaerophilic conditions using the Total RNA Extraction Kit (RBC Biosciences) as was described before [38]. After treatment with DNaseI, cDNA was obtained using random hexamers (Promega) and AMV retrotranscriptase following the manufacturer's instructions. At least three independent cultures were analyzed for each condition. RT qPCR was performed using a LightCycler (DNA Engine M.J. Research) and Real Time PCR mix (EvaGreen qPCR Mix Plus, no Rox). *alkB* gene was analyzed using the following primers: 5′AACTACMTCGARCAYTACGG 3′ and 5′ TGAMGATGTGGTYRCTGTTCC 3′. Validation of RNA-seq results for selected genes was performed with the following primers: *flgA* 5′ACTGTTCAGGGATGTGGTGG′3 and 5′ GCTTCTCCGGGCATTTTCAC′3; *azu* ′5′GATCGA CAAGAGCTGCAGGA′3 and 5′AGAAACCCGTAGTCC GTACCC ′3 and *argC* 5′CGCAAAGTCTTGGTGTGCC′3 and 5′TCCAGTGCTTCTGGAATGC′3. The 16S rRNA gene using primers 5′AGCTTGCTCCTTGATTCAGC′3 and 5′AAGGGCCATGATGACTTGAC′3 was used as reference for normalization of expression levels of target genes in each condition. The cycling conditions were as follows: denaturation at 95 °C for 5 min, 40 cycles at 95 °C for 25 s, 60 °C for 15 s, and 72 °C for 15 s, with the fluorescence acquisition at 80 °C in single mode. Relative changes in the expression of individual genes at aerobic and microaerophilic conditions in the different culture media tested were obtained through the relative standard curve method [20].

Hydrocarbon degradation

Seven-day cultures were analyzed to determine diesel fuel degradation. An uninoculated culture was used as a control and was considered as 100% of remnant hydrocarbon. Remnant hydrocarbons were extracted as described by Tribelli et al. [36]. Briefly, each culture was extracted with 20% v/v *n*-hexane and the solvent phase was dehydrated using anhydrous sodium sulphate. The samples were analyzed by GC using an Agilent 7820A gas chromatograph equipped with a Agilent HP-5 (30 m, 0.32 mm, 0.25 µm) column. 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} and then held for 50 min. For degradation assays, triplicate independent cultures were used. The percentage of residual hydrocarbon was calculated comparing the area of the GC chromatogram of the cultures with those of the control without bacteria. For alkane degradation, the area below each peak was compared. A constant peak with a RT of 26.5 min present in the YPF diesel fuel was use as internal control.

alkB bioinformatic analysis

To characterize the genomic region containing *alkB*, bioinformatic analysis were performed using tools included in the RAST server [4] and Pseudomonas.com site. *alkB* gene promoter region was analyzed using the Virtual Footprint tool available in PRODORIC Database [28]. Transcription binding sites, searched using 500 bp upstream ATG of *alkB* genes belonging to *P. extremaustralis* 14-3b (PE143B_0112385), *P. aeruginosa* PAO1 (PA1525 and PA2574 for *alkB2* and *alkB1*, respectively), *P. protegens* Pf-5 (PFL_2935) and *P. fluorescens* SBW25 (PFLU3535), were compared.

Statistical analysis

The significance of the differences among different conditions was evaluated by the Mann–Whitney test with confidence levels at > 95% (i.e., $P < 0.05$ was considered as significant) [24]. Fisher's test was used for gene over-representation analysis [13].

Data availability

RNA-seq data were deposited in the European Molecular Biology Laboratory under accession number E-MTAB-5440.

Results and discussion

Expression profile under microaerophilic conditions in *P. extremaustralis*

The RNA expression profile of *P. extremaustralis* cultures growing under microaerophilic or aerobic conditions in LB cultures supplemented with KNO_3 revealed 5977 transcripts, including 187 putative regulatory RNAs. Rockhopper software analysis allowed the identification of genes differentially regulated at micro-aerobiosis (relative to aerobiosis) with statistical relevance. Under low oxygen conditions 357 genes were down-regulated and 337 were up-regulated ($P < 0.05$ and $Q < 0.05$, Table S1 and S2). This technique also revealed the existence of 53 novel intergenic sRNAs with differential expression.

To validate RNA-seq results a comparison with RT qPCR assay of some randomly selected genes (*flgA*, *azu* and *argC*) was performed. The genes *flgA*, *azu* and *argC* codify for the flagellar basal body P-ring biosynthesis protein FlgA, azurin and *N*-acetyl-gamma-glutamyl-phosphate reductase, respectively. While *flgA* and *azu* showed correlation between both analytical techniques (Table S3), in *argC*, both RNA-seq

and RTqPCR assays presented low expression under microaerophilic conditions although the RT qPCR experiments showed significant differences (Table S3) In addition, RT qPCR experiments of other genes previously studied in *P. extremaustralis* under microaerophilic conditions was compared with the RNA-seq profile. These genes included *phaC*, encoding a polyhydroxybutyrate (PHB) synthase [39], and *ccoN* and *cioA*, both low oxygen affinity cytochromes [40] which were up-regulated, down-regulated or without changes, respectively, under microaerophilic conditions showing a concordance between both techniques.

To analyze RNA-seq results, differentially expressed genes were classified by functions (Fig. 1, Table S1 and S2). Genes involved in anaerobic metabolism (denitrification, arginine and pyruvate fermentation), transport and secretion, and different oxygenase and dioxygenase proteins as well as biological regulation were found to be up-regulated. Additionally, genes encoding regulatory proteins involved in low oxygen response, including *narL*, *aer* and bacterial chemotaxis related genes *cheY*, *cheX*, *cheW* and *cheA* were up-regulated as well. In contrast, genes involved in aminoacid metabolism, ethanol oxidation, regulator proteins and drug and metal resistance were down-regulated. To determine if some functions were over-represented in the up-regulated or the down-regulated differentially expressed gene data set, Blast2GO analysis was carried out. For down-regulated genes, amino acid metabolism and metal binding categories were over-represented (Fisher's test using Blast2GO, $P < 0.05$, Fig. S2a). Blast2GO analysis of the up-regulated genes showed that genes encoding oxygen binding proteins, regulatory and signal transduction elements and kinase activity constituted the over-represented categories (Fig. S2b).

These results are aligned with those reported in *P. aeruginosa* growing under anaerobic conditions [40], where the activation of fermentation and alternative respiratory chain (denitrification) processes, together with the down regulation of the genes involved in translation and biosynthesis of aminoacids were observed.

Surprisingly, *P. extremaustralis* genes involved in *n*-alkane degradation were up-regulated under microaerophilic conditions (Table S1).

Genetic organization and expression of hydrocarbon degradation genes under microaerophilic conditions

Alkane degradation related genes were widely studied in *P. putida* Gpo1 and in *P. aeruginosa* PAO1 [1, 11, 21, 34]. Whereas *P. putida* Gpo1 presents the alkane degradation genes placed in a plasmid, *P. aeruginosa* PAO1 harbors in

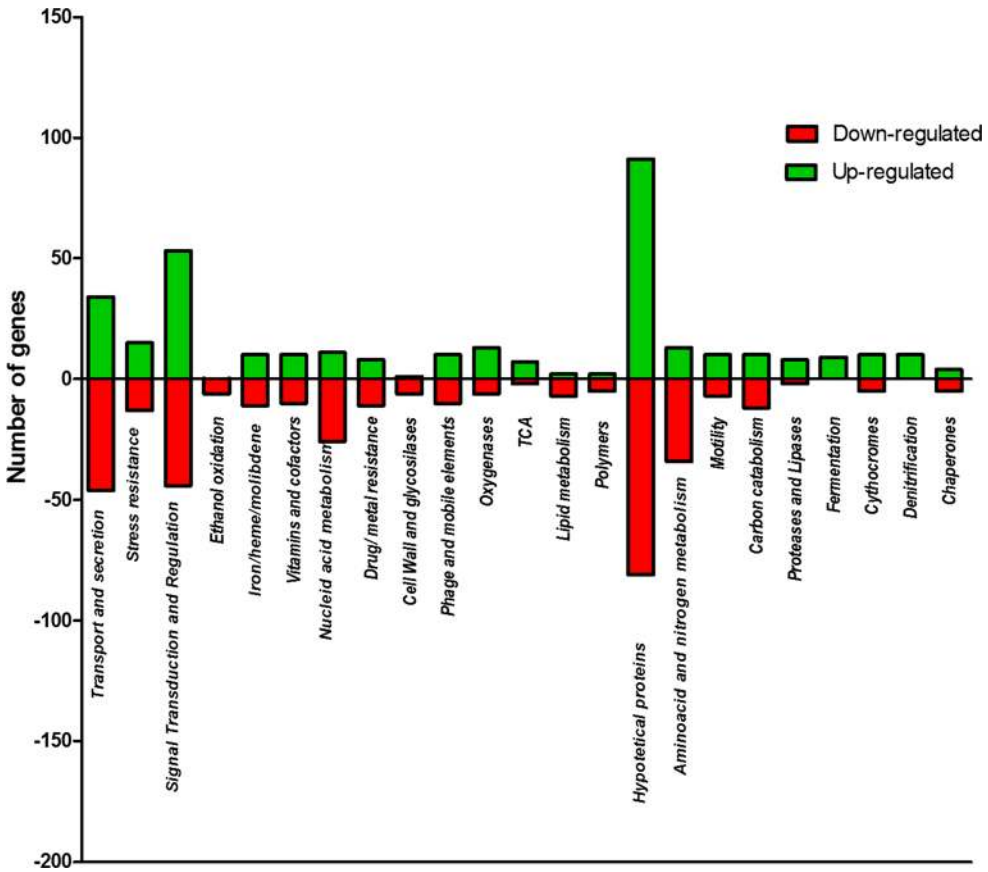

Fig. 1 Classification of the significant differentially expressed genes under microaerophilic conditions into functional categories (Rockhopper $P < 0.05$ and $Q < 0.05$). Green and red bars represent up- and down-regulated genes, respectively (color figure online)

its chromosome two different alkane monooxygenase, one hydrocarbon degradation facilitator protein coding gene and other ubiquitous genes related to alkane degradation such as rubredoxine (*alkG*), rubredoxine reductase (*alkT*), aldehyde dehydrogenase and alcohol dehydrogenase and those belonging to β-oxidation pathway. In *P. extremaustralis*, genes involved in alkane degradation were located at different chromosomal positions. A putative operon composed by *alkB* and two genes encoding protein activators for alkane oxidation (*praA* and *praB*) were present in *P. extremaustralis*. This genetic organization was also found in *P. protegens* Pf-5, and *P. fluorescens* SBW25, two closely related species to *P. extremaustralis* (Fig. 2). *alkS*, a gene encoding a positive regulator of the *alkBFGHJKL* operon and present in the OCT plasmid in *P. putida* Gpo1 [6] was not found in *P. extremaustralis*' genome. Surprisingly, *P. extremaustralis* genes involved in *n*-alkane degradation were expressed differently in microaerophilic conditions in LB cultures compared with aerobic cultures. The *alkB* gene, encoding the key enzyme for alkane degradation, alkane 1-monooxygenase, *praA* and *praB* encoding hydrocarbon facilitating proteins, and other genes related to this pathway, such as those encoding alcohol and aldehyde dehydrogenases, were up-regulated under low oxygen conditions (Fig. 3, Table S1). Additionally, genes involved in steps of fatty acid β-oxidation were also up-regulated, while rubredoxin coding genes (*alkG* and *alkT*) necessary for the oxidation reaction of alkanes were expressed similarly in aerobic and microaerophilic conditions (Fig. 3). A transcriptional study in *P. aeruginosa* PAO1, comparing the expression profile in jet fuel and glycerol supplemented cultures, showed that both *alkB1* and *alkB2* were induced in presence of jet fuel, although at different levels [16]. A quantitative proteomic study in this bacterium showed that AlkB2 was highly expressed in response to octadecane as carbon source in contrast to AlkB1, which was expressed equally in presence and absence of octadecane [22]. RT qPCR for *alkB* gene expression analysis was performed in minimum medium supplemented with diesel fuel as carbon source, with and without KNO_3 under both aerobic and microaerophilic conditions. The latter resulted in an over-expression of the *alkB* gene compared with aerobic conditions (Fig. 4). Although a higher fold change was observed in KNO_3 supplemented cultures, this difference resulted not significant (Fig. 4, Mann–Whitney test, $P > 0.05$). The promoter region of the *alkB* gene was analyzed in silico in *P. extremaustralis* and aligned with the corresponding regions from *P. aeruginosa* PAO1, *P. protegens* Pf-5 and *P. fluorescens* SWB25. A putative binding site for the anaerobic global regulator, Anr, was identified upstream of the *alkB* genes in *P. extremaustralis* and *P. fluorescens* SWB25 and of *alkB1* in *P. aeruginosa* PAO1 (Fig. 2) suggesting that this microaerophilic degradation pathway could be extended to others alkane degraders *Pseudomonas*. Anr is a global regulatory protein that controls the transition from aerobiosis to micro-aerobiosis as well as the expression of genes involved in anaerobic metabolism [32, 35, 37, 38, 40, 41]. The presence of an Anr-box in the promoter region of the *alkB* gene was in line with both the higher expression under low oxygen conditions in *P. extremaustralis*, and the transcriptional data of *P. aeruginosa* PAO1 showing higher expression of *alkB1* in hypoxia [2].

Hydrocarbon degradation under low oxygen conditions

The up-regulation of the *alkB* gene observed in LB cultures without hydrocarbon under microaerophilic conditions, and the presence of an Anr-box like in the promoter zone of *alkB*, led to hypothesize that micro-aerobiosis was the preferred condition that allowed *P. extremaustralis* to grow using diesel fuel as the sole carbon source. Hydrocarbon degradation was analyzed in aerobic and microaerophilic cultures in minimal medium supplemented with diesel fuel as carbon source.

Pseudomonas extremaustralis was able to grow under microaerophilic conditions using diesel fuel as the sole carbon source, consuming 15.95 ± 2.97% of the diesel fuel

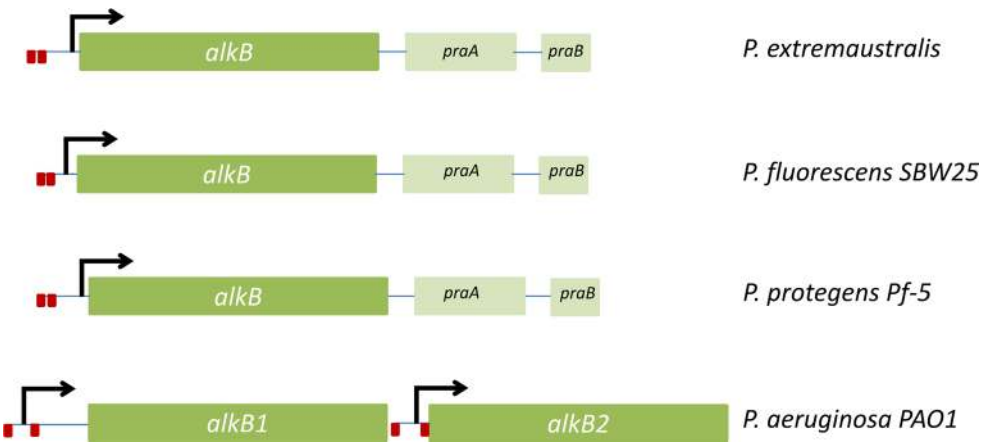

Fig. 2 Genetic organization of genes coding for alkane degradation in *P. extremaustralis* and other *Pseudomonas* species. Arrows indicate the direction of gene transcription and the relative size of each open reading frame (ORF). Red squares indicate putative Anr binding sites (color figure online)

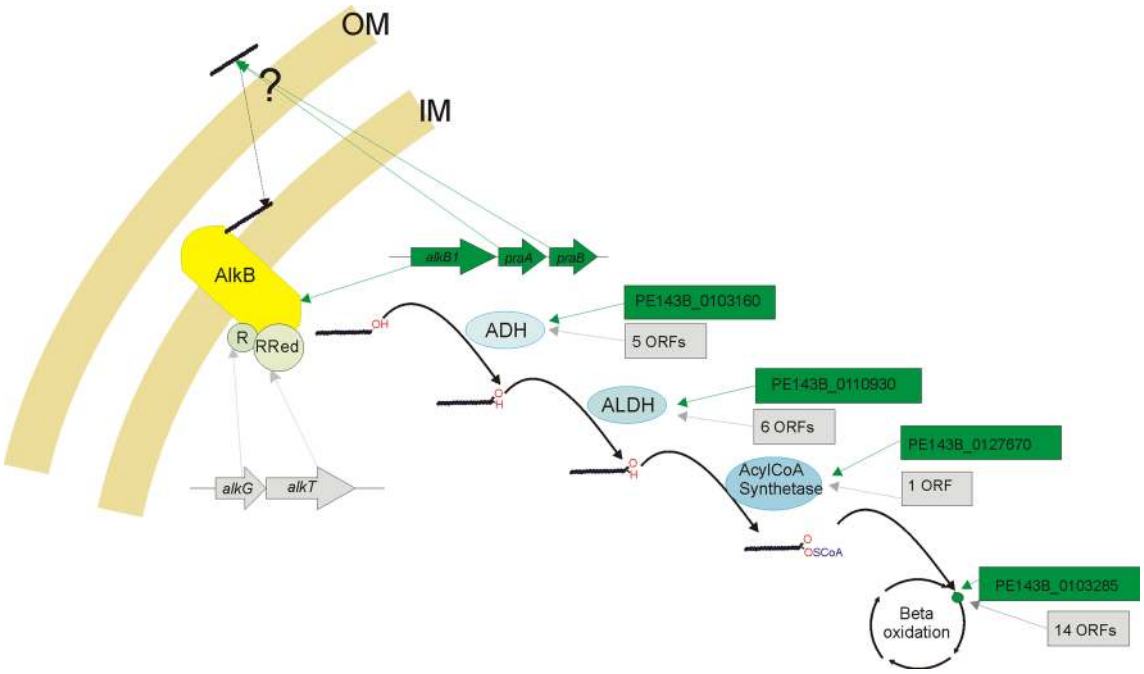

Fig. 3 Alkane degradation and the ß-oxidation pathway in *P.extremaustralis*. Gray arrows and boxes represent genes without differences in their expression. Green arrows and boxes indicate up-regulated functions under low oxygen tension. *OM* outer membrane, *IM* inner membrane, *AlkB* alkane monooxygenase B, *R* rubredoxin, *RRed* rubredoxin reductase, *ADH* alcohol dehydrogenase, *ALDH* aldehyde dehydrogenase. Gene name and its respective *locus* tag in *P.extremaustralis* are indicated (color figure online)

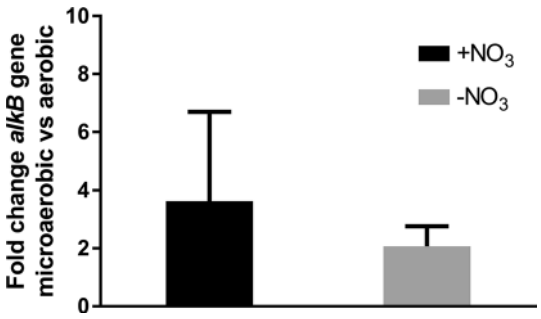

Fig. 4 Quantitative Real Time PCR: total RNA was extracted from *P. extremaustralis* cultures grown at microaerophilic and aerobic conditions with and without KNO_3. *alkB* expression was normalized using rRNA 16S gene expression for each condition and a ratio between aerobic and microaerophilic condition was calculated. Values represent mean ± standard deviation (SD) of three independent experiments

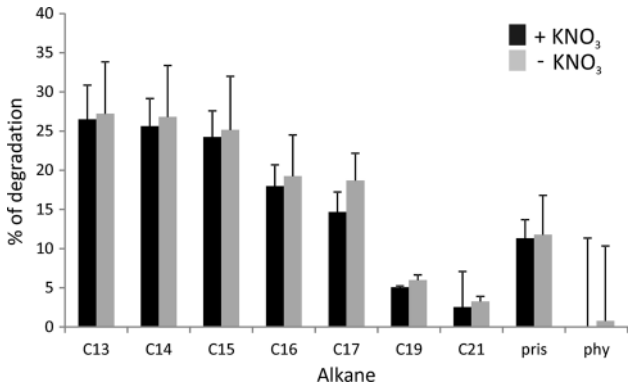

Fig. 5 Comparison of alkane utilization by *P. extremaustralis* growing at low oxygen tension with and without KNO_3. Degradation of carbon compound of different chain length relative to an uninoculated control measure by GC is shown. Bars represent mean ± SD of three independent experiments

after 7 days of growth. However, under aerobic conditions no hydrocarbon degradation was observed. These results are aligned with those previously observed in biofilm cultures [36]. The degradation under low oxygen conditions involved the C13–C19 aliphatic hydrocarbon fraction, including branched hydrocarbons as pristane, but not phytane (Fig. 5). To analyze the possibility that intra-aerobic hydroxylation during denitrification [8] could be used by *P. extremaustralis*, alkane degradation with and without the addition of KNO_3 was tested. No differences in alkane degradation were observed under those conditions (Fig. 5).

To study the effect of diesel fuel degradation on *P. extremaustralis* growth, CFU/ml were calculated after 7 days in both aerobic and microaerophilic cultures, including control cultures without carbon source (Fig. 6). There was no difference in viable bacterial counts under aerobic conditions between cultures supplemented with diesel fuel and those without carbon source, being the result in agreement

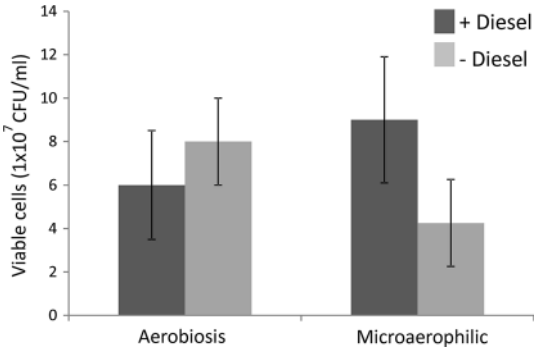

Fig. 6 Survival under aerobic and microaerophilic conditions in diesel fuel supplemented cultures. CFU/ml number was determined by colony plate count assay. Bars represent mean ± SD of three independent experiments

with the absence of alkane degradation in this condition. Under microaerophilic conditions, a slight increment in CFU/ml (Mann–Whitney test, $P = 0.07$) was observed with diesel fuel as carbon source in comparison with control cultures. The CFU/ml increment in diesel fuel supplemented microaerophilic cultures together with the low alkane degradation led to hypothesize that, the use of alkanes in microaerophilic conditions tends to support cellular viability more than bacterial replication. To test this hypothesis, a long-term culture (30 days) was analyzed under microaerophilic conditions showing again a small significant increase (Mann–Whitney test, $P = 0.04$) in CFU/ml in the diesel fuel supplemented media relative to the control without carbon source reaching a value of $2.6 \times 10^6 \pm 2.7 \times 10^5$ CFU/ml and $1.2 \times 10^6 \pm 2.610^5$ CFU/ml, respectively. As previously described [12], *Pseudomonas* species are able to use, under microaerophilic conditions, some metabolic pathways like pyruvate fermentation allowing survival for prolonged periods (18 days) without active growth. In this work not only an increase in *P. extremaustralis* survival but also a slow active growth was observed. Overall, the transcriptomic analysis allowed the identification of the unexpected over-expression under low oxygen conditions of necessary genes for hydrocarbon degradation in LB cultures. Our results showed that in *P. extremaustralis*, *alkB* expression, and alkane degradation are linked and dependent on the oxygen tension. The presence of an Anr-box like in the promoter region of the *alkB* gene suggests the microaerophilic induction of this gene. Interestingly, *alkS* the positive regulator of alkane degradation in *P. putida* Gpo1 [6, 17, 18], is absent in *P. extremaustralis* as well as in other *Pseudomonas* species such as *P. aeruginosa* PAO1 [34]. Additionally, it has been reported that in *P. putida* Gpo1 *alkB* expression is influenced by the level of expression of the cytochrome ubiquinol oxidase in addition to catabolic control [11]. The results showed not only a higher expression of *alkB* and other relevant genes but also diesel fuel degradation under conditions of oxygen limitation. Moreover, experiments performed in diesel fuel and glucose supplemented cultures showed similar alkane degradation (16.9 ± 3.8%) after 7 days in line with the hypothesis of other conditions rather than carbon source as the main regulatory factor in *P. extremaustralis*. The alkane degradation capability of *P. extremaustralis*, a bacterium isolated from a pristine environment, could be related to the fact that alkanes can be produced by different organisms from fatty acid metabolites, and found, for example, as components of plant cuticle waxes, insect pheromones and also in microorganisms such as cyanobacteria [33].

Interestingly, our results suggest that alkane degradation could be important for long-term survival and slow growth of this bacterium using diesel fuel as carbon source under microaerophilic conditions. Microaerophilic conditions could be found in a recent oil contaminated environment, where pollutants generate a strong impact in habitat quality affecting different environmental properties such as water flow, oxygen availability, nutrients, and light access [33]. As already described, when an oil spill occurs, a change in microbial communities' composition begins [26]. In the Deepwater Horizon oil spill, aliphatic hydrocarbons degrading bacteria with large duplication times and more generalist strategies were the first ones to react to the environment change, being further replaced by most active degraders [29].

This study provides evidence of the activation of the alkane degradation pathway under microaerophilic conditions, regardless the presence of environmental hydrocarbon in a bacterium isolated from a pristine environment. This metabolism could represent an adaptive advantage in a changing environment by supporting survival under low oxygen conditions as found in recent oil contaminated sites. The role of this kind of microorganism should be considered as an important factor for the design of bioremediation strategies.

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