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Research Paper

Transcriptional profiling of genes involved in *n*-hexadecane compounds assimilation in the hydrocarbon degrading *Dietzia cinnamea* P4 strain

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Abstract

The petroleum-derived degrading *Dietzia cinnamea* strain P4 recently had its genome sequenced and annotated. This allowed employing the data on genes that are involved in the degradation of *n*-alkanes. To examine the physiological behavior of strain P4 in the presence of *n*-alkanes, the strain was grown under varying conditions of pH and temperature. *D. cinnamea* P4 was able to grow at pH 7.0-9.0 and at temperatures ranging from 35 °C to 45 °C. Experiments of gene expression by real-time quantitative RT-PCR throughout the complete growth cycle clearly indicated the induction of the regulatory gene *alkU* (TetR family) during early growth. During the logarithmic phase, a large increase in transcriptional levels of a lipid transporter gene was noted. Also, the expression of a gene that encodes the protein fused rubredoxin-alkane monooxygenase was enhanced. Both genes are probably under the influence of the AlkU regulator.

Key words: *Dietzia cinnamea*, *n*-hexadecane assimilation, *alk* genes, rubredoxin-alkane monooxygenase, real-time quantitative RT-PCR.

Introduction

Petroleum-derived hydrocarbons cover a wide range of linear alkanes of different sizes, cycle-alkanes and aromatic hydrocarbons of natural origin. Microorganisms able to degrade such hydrocarbons have received greater attention in recent years. This is mainly due to their potential for bioremediation of oil spills or applications in biotransformations of fine chemicals (Atlas and Atlas, 1991). The utilization of aliphatic alkanes by bacteria is driven by two important steps: the transport of alkane compounds into the cell and a first reaction catalyzed by a monooxygenase, which converts alkanes to alkanols, also called hydroxylases (van Beilen and Witholt, 1994). These enzymes constitute a set of related non-heme iron integral membrane oxygenases, collectively named alkane hydroxylase (AlkB) (Smits *et al.*, 1999). Currently there are three categories of alkane hydroxylases (van Beilen and Funhoff, 2007), *i.e.* methane monooxygenase, active on short-chain alkanes (C1-C4), membrane-bound non-heme iron and cytochrome P450 monooxygenases, which act on medium-chain alkanes (C5-C16) (van Beilen and Witholt, 1994) and poorly-characterized hydroxylases that oxidize long-chain alkanes (> C16) (Feng *et al.*, 2007; Throne-Holst *et al.*, 2007). The complete biochemical pathway responsible for assimilation of n-alkanes is most extensively described in *Pseudomonas putida* Gpo1 (van Beilen *et al.*, 2001). In this species, the genes are arranged in the *alkBFGHJKL* operon localized in the OCT plasmid, containing all genes involved in hydrocarbon degradation (Van Hamme *et al.*, 2003).

While there are several studies on the hydroxylation of *n*-alkanes by monooxygenases, the understanding of the acquisition of such hydrophobic compounds remains difficult. The low solubility of alkanes raises the question about the best strategies adopted by the cell for the acquisition of

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such compounds. Selective and active transport, which is energy-dependent, has been suggested by Beal and Betts (2000), who used an inhibitor of cytochrome oxidase to block oxidative phosphorylation, confirming the reduction in *n*-hexadecane uptake. A possible membrane lipoprotein (Blc) is suggested to be directly involved in the transport of alkanes to the inside of cells of *Alcanivorax borkumensis* (Sabirova *et al.*, 2011). Another possible process involved in the acquisition of *n*-alkanes is the biosynthesis of biosurfactant, which apparently increases the solubility of *n*-alkanes through changes in its physical behavior (Van Hamme and Ward, 2001).

In recent years, at least 60 genera of aerobic bacteria and five anaerobic bacteria have been described to be able to degrade hydrocarbons of petroleum (Sakai *et al.*, 1994; Prince, 2005). Among these bacteria, we find *Rhodococcus* (Van Hamme and Ward, 2001), *Alcanivorax* (Liu *et al.*, 2010) and *Pseudomonas* (Zhang *et al.*, 2011). Recently, species of *Dietzia* have emerged as potential degraders of petroleum-derived compounds. *Dietzia* spp. have been isolated from diverse environments, such as tropical soil (von der Weid *et al.*, 2007), soda lakes (Duckworth *et al.*, 1998), oil fields (Borzenkov *et al.*, 2006), deep-sea sediments (Colquhoun *et al.*, 1998), skin and intestinal tracts of marine fish (Yumoto *et al.*, 2002) and decomposing reed rhizomes (Borsodi *et al.*, 2005). In addition, studies of *n*-alkane degradation by *Dietzia* species have shown the ability to use different compounds (C_6-C_{40}) (Bihari *et al.*, 2011; Wang *et al.*, 2011). In a previous study of the effect of biostimulation on the diversity of the bacterial community present in a sandy loam (Cambisol) soil with no history of previous contamination, *Dietzia cinnamea* strain P4 was isolated (Evans *et al.*, 2004). Subsequent studies of its biodegradation potential of petroleum-derived compounds revealed that strain P4 is able to grow on different sizes of linear and aromatic hydrocarbons (von der Weid *et al.*, 2007).

Recently, the *D. cinnamea* P4 genome has become available in the NCBI database under the access number NZ_AEKG01000000. This genome is the first genome of a *Dietzia* species that has been sequenced and annotated. The genome annotation identified several enzymes of biotechnological interest and confirmed the presence of the genes involved in hydrocarbon compound degradation, *e.g. n*-alkanes, biphenyl and benzene compounds (Procópio *et al.*, 2012). Here, we described the detailed genetic organization of these genes in the strain P4 chromosome, and the profile of degradation of *n*-hexadecane compounds, assessing the transcript levels of *alk* genes during growth by real-time RT-PCR.

Materials and Methods

Chemicals and oligonucleotide primers

Bushnell-Haas mineral salts medium (BH) was purchased from Difco, BD (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). *n*-Hexadecane (99% pure) and glucose were purchased from Sigma Co (St. Luis, MO, USA). Luria-Bertani Agar (LB-agar, tryptone 1%, NaCl 0.5%, yeast extract 0.5%, agar 1.5%) medium was obtained from Deutche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany. The primers used for the amplification of the interested genes are described in the Table 1.

Bacterial strain and growth conditions

All experiments were conducted with *Dietzia cinnamea* P4 strain in aerobic conditions. From a single colony, previously grown on LB-agar, a pre-inoculum was prepared in 50 mL Erlenmeyer flasks containing 10 mL of sterile BH medium (pH 7.0) supplemented with 1 g/liter of glucose (wt/vol) or 1% of *n*-hexadecane (vol/vol), at 28 °C (under agitation - 120 rpm) up to the late-exponential phase of growth. The *n*-hexadecane compound was filter-sterilized using a solvent-resistant Whatman PVDF sterile syringe filter. Then 1 mL of each pre-inoculum was transferred in 250 mL Erlenmeyer flasks containing 100 mL of BH medium supplement with 1 g/liter of glucose (wt/vol)

Table 1 - Primers used in this study.

Primer	Sequence	Reference or source
alkU-Fwd	5'-ATG CCG ATG CGG GCG ATG-3'	This study
alkU-Rev	5'-TGT TCG AGG CCA TGC ACG-3'	This study
alkB-rub-Fwd	5'-CCC AGT CAC GAC GTT GTA AAA CG-3'	This study
alkB-rub-Rev	5'-AGC GGA TAA CAA TTT CAC ACA GG-3'	This study
lipid transporter-Fwd	5'-TCC TCA TCC TCT CCG TCT TC-3'	This study
lipid transporter-Rev	5'-CGG TCA TCT GGT CGT TCA TC-3'	This study
Acox1-Fwd	5'-TCT CGG TCA TGG CGA AGG AG-3'	This study
Acox1-Rev	5'-CGC GGA CGA CAC TCC GTA TT-3'	This study
16S-Fwd (U968)	5'-AAC GCG AAG AAC CTT AC-3'	(Nübel *et al*., 1996)
16S-Rev (L1401)	5'-CGG TGT GTA CAA GAC CC-3'	(Nübel *et al*., 1996)

or 1% of *n*-hexadecane (vol/vol), following the same conditions described above. In order to evaluate the growth in different pHs, the P4 strain was cultivated in BH medium at pH 5.0, 7.0 and 9.0, previously adjusted with HCl or NaOH and supplemented with 1% of *n*-hexadecane (vol/vol). The cultures were grown in three replicate flasks during 10 days. Sampling (1 mL) was done every day, centrifuged at 8,000g for 4 min, then the cell pellets were washed twice in 1 mL of sterile BH, and then optical density was measured at 660 nm to determine its growth curve.

Quantitative real-time PCR

One mL of each culture containing *n*-hexadecane as the sole carbon source was taken 2 hours after the onset of growth (early phase), in the late-exponential and mid-stationary phases, and growth in glucose as sole carbon source was taken only in late-exponential phase. Total RNA was extracted using the RNeasy Mini kit (Quiagen, Valencia, CA, USA) according to the manufactures protocol with the addition of one initial step of nitrogen liquid for cell lyses due to the difficulty of breaking the cell wall of P4 strain. Then the total RNAs were treated with DNase I (Promega, São Paulo, Brazil). The integrity of the RNAs was checked by agarose gel electrophoresis and the yield was estimated using a Nanodrop UV spectrometer (Thermo Scientific, Wilmington, DE, USA). About 100 ng of RNA from each sample were used for cDNA synthesis using random hexamers by the cDNA Synthesis kit (Bioline, Boston, Ma, USA) according to the manufactures protocol. The yield of cDNAs was estimated by Nanodrop UV spectrometer and their concentrations adjusted to about 1 ng. For real-time quantitative RT-PCR, 2 μL of cDNA was mixed with Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 0.2 μg of forward and reverse primers of each gene to by analyzed (Table 1) in a final volume of 25 μL in three replicates. In addition, no-templates controls, in three replicates for each gene to by analyzed, also were included. Expression of the 16S rRNA gene (Table 1) was used as a reference gene to normalize tested genes and was correlated to the amount of corresponding transcripts in samples grown on glucose. The real-time quantitative RT-PCR reaction was carried out with the ABI Prism 700 Sequence Detection System (Applied Biosystems), following the protocol: one cycle at 95 °C for 10 min, 40 cycles at 95 °C for 1 min, followed by 60 °C for 1 min, 72 °C for 2 min. The specificity of the amplification was verified at the end of the PCR run through uses of the ABI Prism Dissociation Curve Analysis software, and the normalized relative fold change in mRNA levels were calculated for the gene of interest in each sample using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Sequence analysis

Sequence analysis of interesting genes and visualization of their organization in the strain P4 genome was performed using the Artemis program (Rutherford *et al.*, 2000). Searches for conserved domains were performed using the BLAST suite (Altschul *et al.*, 1990) and the transmembrane domains were identified using THMMH (Krogh *et al.*, 2001). Alignments between the deduced amino acid sequences were performed by Clustal W (Thompson *et al.*, 2002). Phylogenetic analysis of the analyzed genes were structured by maximum-parsimony method using the MEGA4 program (Tamura *et al.*, 2007) and bootstrapping analysis was used to evaluate the tree topology by 1000 re-samplings.

Results

Genetic analysis of *alk* genes

The genome annotation of *D. cinnamea* P4 enabled the identification of a DNA region of 14.9 kbp, which contained 15 ORFs (access number: NZ_AEKG01000061), three of which are annotated as directly involved in the assimilation of aliphatic hydrocarbons (Procópio *et al.*, 2012). ORF5 (ZP_08022272), with 1860 bp, was recorded initially as a putative ABC transporter permease. Subsequent analysis of similarity indicated 99% of homology of this gene with the putative ABC transporter of *Dietzia* sp. E1. The program THMMH identified five transmembrane domains. Next, ORF6, annotated as an alkane 1-monooxygenase (ZP_08022271), with 1527 bp, revealed upon subsequent analysis 98% of homology with an alkane hydroxylase/rubredoxin fusion protein. This protein contains six transmembrane domains. ORF7 was annotated as a putative TetR family transcriptional regulator (ZP_08022270), with 625 bp. The probable arrangement of these genes on the chromosome of strain P4, with the presence of *alkB-rub* natural fusion genes, is very similar to that previously described in the related *Dietzia* sp. E1 (Figure 1). The putative alkane monooxygenase hydroxylase gene (*alkB-rub*, ORF6) is arranged in fusion with a rubredoxin gene. Thus, the probable *alkB-rub* gene is flanked by one TetR family transcriptional regulator (ORF7), which is described as *alkU,* in other degrader bacteria, and a putative bifunctional ABC lipid transporter (ORF5).

Multiple alignments between the full-length sequences of *D. cinnamea* P4 (ZP_08022271), *Dietzia* sp. E1 (ACN62569), *Dietzia* sp. DQ12-45-1b (AEM66514), *Prauserella rugosa* NRRL B-2295 (CAB51024), *Nocardioides* sp. CF8 (AAK31348), and the fragment sequences of *Dietzia* sp. K44, *Dietzia* sp. ITRH 56 and *Dietzia* sp. H0B, showed great similarity between the sequences of P4, H0B and E1 (about 95%), and lower levels with K44 and ITRH56, 63% and 44% respectively. In addition, the multiple alignments between the deduced amino acid sequences identified three His boxes (Figure 2a-c) and an additional HYG-motif (Figure 2d). Both are commonly found among the alkane hydroxylases and have essential roles in its activity. Next to this, we found two CXCG Rub

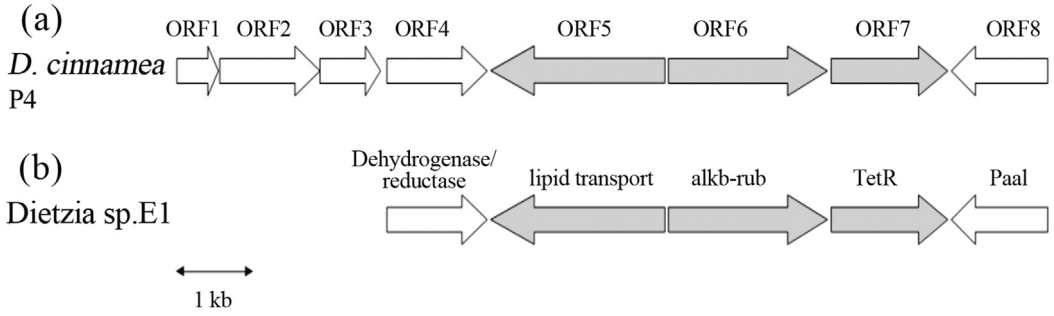

Figure 1 - Genetic organization of alkane degradative genes from *Dietzia cinnamea* strain P4 aligned to homologue segment from *Dietzia* sp. E1 (Bihari *et al.*, 2011). (a) *Dietzia cinnamea* P4 (ORF1) hypothetical protein gene, (ORF2) N-acyltransferase gene, (ORF3) tryptophan synthase beta-subunit gene, (ORF4) short-chain alcohol dehydrogenase gene, (ORF5) bifunctional ABC lipid A transporter gene, (ORF6) alkane 1-monooxygenase gene, (ORF7) TetR-type family transcriptional regulator gene and (ORF8) PaaI, thioesterase gene. (b) Organization genes of *Dietzia* sp. E1.

sequence motifs (Figure 2e, f), indicating an AlkG-type rubredoxin proteins (van Beilen *et al.*, 2002, 2003).

In order to analyze the phylogenetic relationship between the deduced amino acid sequence of putative AlkB of P4 strain with other AlkB sequences of related actinobacteria, we compared different classes of alkane monooxygenase hydroxylase sequences available in NCBI database. The phylogenetic tree obtained (Figure 3) showed that the AlkB sequence of strain P4 is positioned in between other alkane monooxygenase sequences of *Dietzia* strains, separated from AlkB sequences of other actinomycetes, which is supported in 100% of the neighbour-joining trees generated. However, the evolutionary distance between the strain P4 AlkB sequence is apparently shorter with AlkB1 and AlkB2 than with AlkB3, AlkB4 and AlkB5. The robustness of the branching pattern tree was supported by 98% of maximum-parsimony and 100% of maximum-likelihood.

Growth and utilization of n-hexadecane as the sole carbon source

We evaluated the growth of strain P4 at different pHs (4.0, 5.0, 7.0 and 9.0), and temperatures (35 °C and 45 °C), using *n*-hexadecane as the sole carbon source (See Materials and methods). The time course of growth of strain P4 is shown in Figure 4. The strain was unable to grow in pH 4.0 and 5.0 at both temperatures; however it showed obvious growth at pH 7.0 and 9.0, at both temperatures. The strain showed initially slow growth, not presenting a significant increase up to 90 h of incubation, whereas after 90 h growth was exponential up to 120 h. Then, a stationary phase commenced, which lasted until 240 h, when cell viability started to decline. Similar growth kinetics was also described for growth in glucose as the sole carbon source at pH 7.0 and 35 °C.

Expression of genes involved in the assimilation of *n*-hexadecane

The expression of putative *alkU*, *alkB-rub,* the bifunctional ABC lipid transporter and acyl-CoA oxidase genes under the induction of *n*-hexadecane substrate during growth were analyzed by real-time RT-PCR. The level of transcripts of interest was compared to that obtained under induction by glucose as the sole carbon source, and the 16S rRNA gene was used for normalization for all conditions used (See Materials and methods). In the early stage of growth (the lag phase), the putative *alkU* gene showed the highest transcript level between the genes analyzed, being 43-fold that observed in the presence of glucose (Figure 5). In contrast, during the logarithmic growth phase, the level of the *alkU* regulator gene dropped to 11-fold, and to -0.5 in stationary phase.

The expression of *alkB-rub* and the bifunctional ABC lipid transporter genes in the presence of hexadecane showed increases of over 47- and 48-fold, respectively, during the late-exponential phase (Figure 5). During the early growth phase, while the alkane-1 monooxygenase-rubredoxin showed a level close to zero, the bifunctional ABC lipid transporter showed 4.6-fold expression. A main destination for *n*-hexadecane in the cell is presumably its use in energy production, which uses the β-oxidation pathway (van Beilen and Witholt, 1994). In order to analyze whether there was any increase in activity of this catabolic pathway, we determined the levels of the *acox* (acyl-CoA oxidase) gene, which plays a key role in lipid metabolism. The expression levels of the *acox* gene during the logarithmic growth phase showed an increase of over 100-fold in the presence of *n*-hexadecane over that with glucose, whereas during the early and mid-stationary phases these expression levels remained low (below zero) (Figure 5).

Discussion

The soil bacterium *D. cinnamea* P4 has previously been isolated from microcosms containing oil-contaminated soil collected from an environmentally protected area of a tropical Atlantic forest (Biological Reserve of Poço das Antas - Brazil) (Evans *et al.*, 2004). Von der Weid *et al.* (2007), in later experiments, showed the ability of strain P4 to grow in a wide range of *n*-alkanes of different sizes (C_{11} to > C_{36}), oil-crude and aromatic hydrocarbons

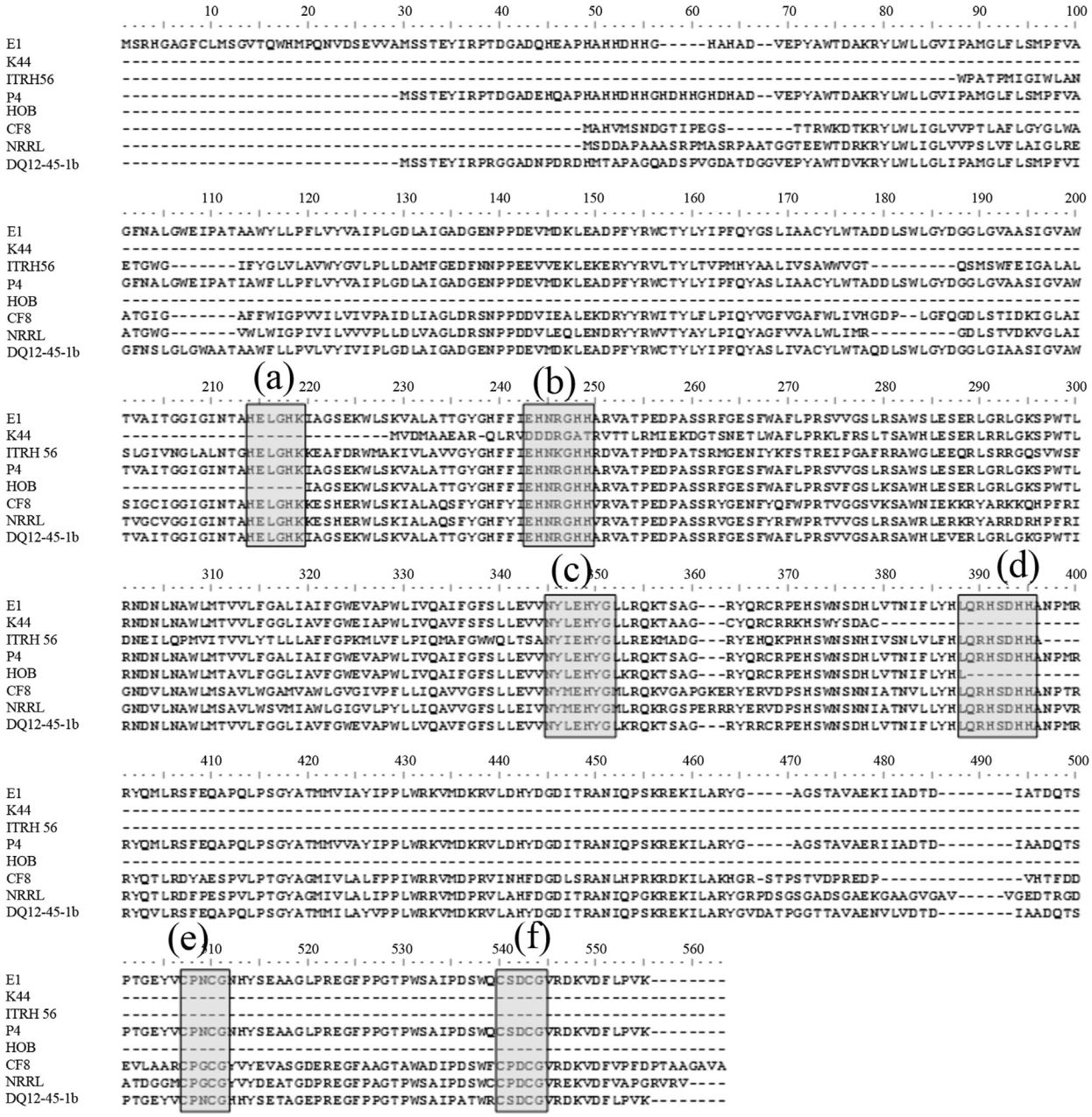

Figure 2 - Multiple alignments of full-length alkanes hydroxylase of (E1) *Dietzia* sp. E1, (acc. ACN62569); (K44) *Dietzia* sp.K44, (acc. AAV68403); (ITRH 56) *Dietzia* sp. ITRH56, (acc. ACH99228); (P4) *D. cinnamea* P4, (H0B) *Dietzia* sp. H0B, (acc. ACR56752); (CF8) *Nocardioides* sp. CF8, (acc. AAK31348); (NRRL) *Prauserella rugosa* NRRL B-2295, (acc. CAB51024) and (DQ12-45-1b) *Dietzia* sp. DQ12-45-1b (acc. AEM66518). Three conserved His boxes (a), (b) and (d), HYG-motif (c) and two CXXCG Rub sequence motifs (e) and (f).

(*e.g.* benzene), revealing its potential to serve as an organism in bioremediation of petroleum hydrocarbons. Recently, the annotation of the genome of the bacterium *D. cinnamea* P4 was released by the NCBI site. This study was the first to describe a draft annotation of a *Dietzia* genome, which makes it highly relevant to advance the field of *Dietzia* biology. The genome revealed several interesting new genes and gene clusters with possible application in biotechnology and confirmed the presence of *alk* genes involved in degradation of *n*-alkanes (Procópio *et al.*, 2012). In the present study, we showed the transcriptional profile of genes involved to use *n*-hexadecane compounds as the sole carbon and energy source under different conditions of pH (7.0-9.0) and temperature (35 °C to 45 °C).

Our bioinformatics analysis described a cluster of genes containing three putative ORFs directly involved in *n*-alkane transport and degradation, which is composed by one putative lipid transporter, one alkane monooxygenase and one TetR transcriptional regulator. The alkane monooxygenase described in this study is fused to a rubredoxin

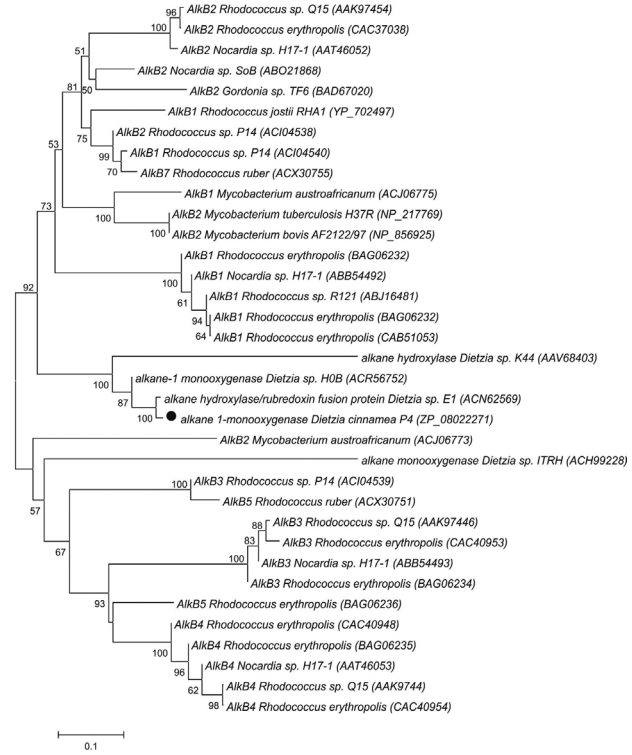

Figure 3 - Neighbour-joining phylogenetic tree based on the alignment of amino acid sequences of related alkane monooxygenase from strains of actinomycetes. GenBank accession numbers are given in parentheses. Only bootstrap values higher 50% out of 1000 replications are shown. Bar represents 0.1 amino acid substitutions per site.

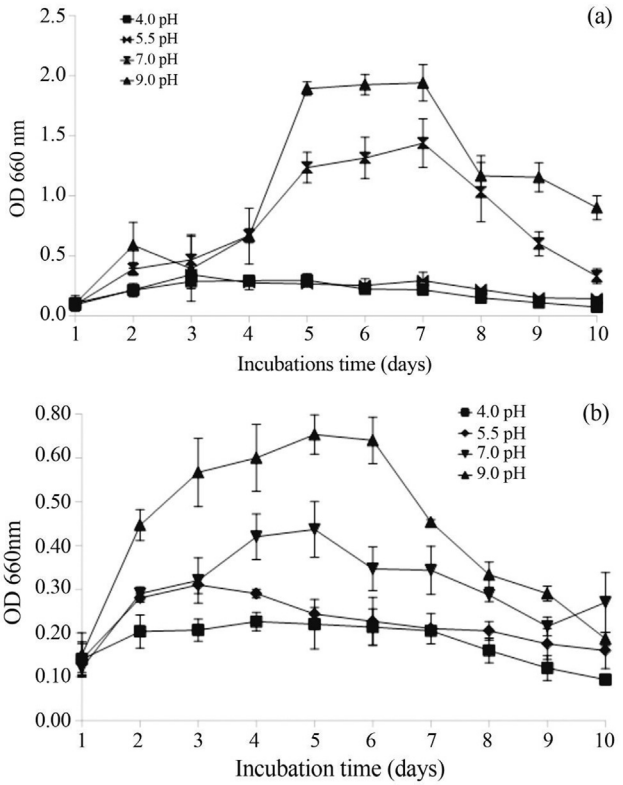

Figure 4 - Growth of *Dietzia cinnamea* P4 on *n*-hexadecane as the sole carbon source in different conditions of pHs (4.0 to 9.0) and temperatures (a) 35 °C and (b) 45 °C.

enzyme, which is necessary for the oxygenation reaction of *n*-alkane compounds. The unusual occurrence of an *alkB-rub* natural fusion gene was also reported in *Nocardioides* sp. CF8 actinomycete (Hamamura *et al.*, 2001) and *Prauserella rugosa* NRRL B-2295 (van Beilen *et al.*, 2002). The presence of a monooxygenase as a fused polypeptide had already been described in *Bacillus* genus (Narhi and Fulco, 1987). The cytochrome P450 fatty acid

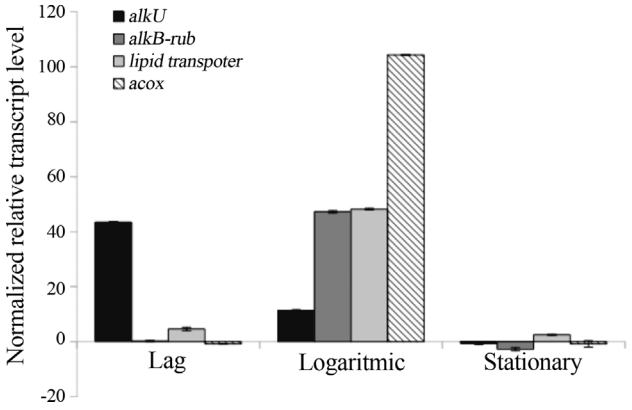

Figure 5 - Expression levels of putative *alkU*, *alkB-rub*, lipid transporter and *acox* genes of *Dietzia cinnamea* P4 strain on *n*-hexadecane as the sole carbon source in different phases of growth. Relative expression levels were determined with real-time quantitative RT-PCR. Data are representative of two independent experiments.

monooxygenase from *Bacillus megaterium* was shown to consist of hydroxylase and reductase components on a single polypeptide (encoded by a single continuous gene), which can be cleaved by trypsin into the respective domains. Recently, the *alkB-rub* natural fusion gene was also reported in *Dietzia* sp. E1 (Bihari *et al.*, 2011). The related *Dietzia* sp. E1 strain has a gene arrangement very similar to that described in *D. cinnamea* P4. In addition to the genes analyzed in this study, we identified other genes in strain P4 that showed homology and similar positions to that found in the chromosome of strain E1.

Although the degradation of differently-sized *n*-alkanes is reportedly ubiquitous in nature by different classes of alkane monooxygenases (AlkBs), the mechanisms have not been completely elucidated. A relationship between the AlkB protein structure and its preference for specific *n*-alkanes has been proposed. In the gram-negative *Pseudomonas putida* Gpo1, the hexagonal structure of AlkB builds a deep hydrophobic pocket, in which four conserved histidine residues allow a selective control over the specificity of the reaction catalyzed by the enzyme (van Beilen *et al.*, 2005). In Gram-positive bacteria, like *Rhodococcus* genus, the presence of multiple alkane hydroxylases is a common feature (Whyte *et al.*, 2002). This feature can be illustrated by the annotation of several *alkB* genes in a single gene cluster, as described in *Rhodococcus ruber* SP2B (Amouric

et al., 2010). Despite the ability to degrade different size of *n*-alkanes, our genome annotation of *D. cinnamea* P4 identified only one putative alkane monooxygenase (Procópio *et al.*, 2012). Phylogenetic analyses positioned the AlkB of strain P4 among those of *Dietzia* species, including the AlkB1 and AlkB2 alkane monoxygenase classes. The close relationship between the strain P4 and E1 AlkB sequences might indicate a similar role in hydrocarbon assimilation in respect of preferred sizes of alkanes (Bihari *et al.*, 2011). The preference for medium- and long-chain hydrocarbons ($\geq C_8$ and $\leq C_{25}$) by *D. cinnamea* P4 was previously described by von der Weid *et al.* (2007).

The analysis of gene expression by real-time RT-PCR provided an excellent tool to determine the level of mRNA in response to environmental signals. Commonly, studies of gene expression of *alk* genes show an instantaneous transcriptional profile, taking into account the levels of transcripts involved in the assimilation of hydrocarbons after (short or long) exposure to the compounds (*e.g.* in late-exponential growth phase). Here, were evaluated the transcript levels of the putative strain P4 *alk* genes during the complete life cycle of the cells of *D. cinnamea* P4. We raised the question as to which levels of *alk* gene expression could be found during the early-phase of growth. In gram-negative bacteria, the regulator identified as the transcriptional regulator of the *alk* operon is *alkS*, belonging to the LuxR family, while in Actinobacteria, the transcriptional regulator identified by the *alk* genes expression is the TetR-type gene (*alkU*). Both regulators are normally found immediately downstream of the *alkB* gene (Whyte *et al.*, 2002). In the annotated genome of *D. cinnamea* P4, the *alk* cluster genes were found in an arrangement where the putative *ulkU* gene is immediately downstream of the *alkB* gene (Procópio *et al.*, 2012). In this study, the expression of the *alkU* gene was analyzed by real-time RT-PCR during the complete growth cycle of P4, in the presence of *n*-hexadecane as the sole carbon source, and compared with the same conditions of growth, but using glucose as only carbon source. The obvious increase in expression levels of the putative *alkU* gene, after two hours in presence of *n*-hexadecane, and the subsequent increase of other *alk* genes in the logarithmic growth phase, indicated a clear role in inducing other *alk* genes, which were later expressed. The regulation of *alk* genes by the transcriptional regulator TetR is poorly described in the literature, and its action has only been related in gram-negative bacteria by the LuxR-type family transcriptional regulator (Moreno *et al.*, 2007, 2009). The most studied transcriptional regulator responsible for *alk* operon induction is the AlkS protein, described in *P. putida* Gpo1 (Whyte *et al.*, 2002). AlkS induces the expression of *alk* genes acting on *n*-alkanes from C_5 to C_{16} (Sameshima *et al.*, 2008). In a study conducted with the E1 strain, the expression levels of the TetR transcriptional regulator were analyzed only in late-exponential phase growth, which detected a low expression level of this gene in the presence of *n*-hexadecane compounds (Bihari *et al.*, 2011).

The results of real-time RT-PCR in our study indicated the clear induction of the putative *alkB-rub* gene by *n*-hexadecane compounds (Figure 5). The increase during the late-exponential phase suggested transcription activated by the TetR-type transcriptional regulator. The hydroxylation reaction performed by alkane monooxygenase-rubredoxin is the key step in the assimilation of *n*-alkanes. Several studies on the induction of *alkB* genes by *n*-alkane compounds have taken into account the sizes of the preferred hydrocarbon chain class of alkane monoxygenases. Analysis of *alkB-rub* expression gene in *Dietzia* sp. E1 with different chain size *n*-alkanes showed the preference for $C \geq 16$ hydrocarbons (Bihari *et al.*, 2011), while in *Gordonia* sp. SoCg had a higher level of *alkB* gene expression when grown in *n*-triacontane than *n*-hexadecane (Lo Piccolo *et al.*, 2011). In addition, although our results did not indicate the possibility of activation of *alk* genes in the presence of hydrocarbons of different sizes, like the *D. cinnamea* P4 strain, the *Dietzia* sp. DQ12-45-1b as well P4 (although they have capabilities to use a wide range of *n*-alkanes) also preferentially degraded medium- and long-chain hydrocarbons (Wang *et al.*, 2011).

Although a probable transmembrane transporter for *n*-alkane compounds remains to be fully elucidated, studies with *Mycobacterium album*, *Rhodococcus erythropolis* S+14He and *Acinetobacter* sp., showed that, in the presence of *n*-hexadecane, there was an accumulation of intracellular inclusion bodies (Kennedy *et al.*, 1975a,b). In addition, from thermodynamics, the presence of intracellular *n*-alkane compounds can only occur by an effective energy-dependent transport system (Kennedy *et al.*, 1975b). In our experiments of real-time RT-PCR, the putative lipid transporter gene of strain P4 showed an increase in expression levels during the late-exponential phase, probably under induction by transcriptional regulator AlkU. However, the expression of the lipid transporter genes during early and stationary phases showed elevated baseline levels when compared with other genes analyzed in this study. This was probably due to the need of such lipid carriers, at basal levels, required for the acquisition of *n*-alkanes compounds in a first contact. The presence of a lipid transporter along with the *alkB* gene is related by Liu *et al.* (2010) in *Geobacillus* sp. MH-1 strain. In addition, the location of a lipid transporter along of other genes involved in the assimilation of *n*-alkane compounds described in *Dietzia* sp. E1, with its concomitant induction by *n*-alkanes compounds, suggest its role in uptake of this compounds of environment (Bihari *et al.*, 2011).

In conclusion, our results show that the P4 strain is able to grow using compounds of *n*-hexadecane as sole carbon source. The analysis of gene expression during the complete cycle growth suggests the role of the putative alkU TetR-family transcriptional regulator in inducing of

the *alkB-rub* and lipid transporter genes during the late-exponential phase. This new information helps to elucidate the physiological behavior of the *alk* pathway, responsible for the degradation of oil-derived, which will allow a better use of this strain in bioremediation and biotransformation technologies.

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