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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Metabolic responses of *Rhodococcus erythropolis* PR4 grown on diesel oil and various hydrocarbons

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Abstract *Rhodococcus erythropolis* PR4 is able to degrade diesel oil, normal-, iso- and cycloparaffins and aromatic compounds. The complete DNA content of the strain was previously sequenced and numerous oxygenase genes were identified. In order to identify the key elements participating in biodegradation of various hydrocarbons, we performed a comparative whole transcriptome analysis of cells grown on hexadecane, diesel oil and acetate. The transcriptomic data for the most prominent genes were validated by RT-qPCR. The expression of two genes coding for alkane-1-monooxygenase enzymes was highly upregulated in the presence of hydrocarbon substrates. The transcription of eight phylogenetically diverse cytochrome P450 (*cyp*) genes was upregulated in the presence of diesel oil. The transcript levels of various oxygenase genes were determined in cells grown in an artificial mixture, containing hexadecane, cycloparaffin and aromatic compounds and six *cyp* genes were induced by this hydrocarbon mixture. Five of them were not upregulated by linear and branched hydrocarbons. The expression of fatty acid synthase I genes was downregulated by hydrocarbon substrates, indicating the utilization of external alkanes for fatty acid synthesis. Moreover, the transcription of genes involved in siderophore synthesis, iron transport and exopolysaccharide biosynthesis was also upregulated, indicating their important role in hydrocarbon metabolism. Based on the results, complex metabolic response profiles were established for cells grown on various hydrocarbons. Our results represent a functional annotation of a rhodococcal genome, provide deeper insight into molecular events in diesel/hydrocarbon utilization and suggest novel target genes for environmental monitoring projects.

Keywords *Rhodococcus erythropolis* PR4 · Simultaneous hydrocarbon biodegradation · Transcriptomics · Diesel oil decomposition · Metabolic response · Oxygenases

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Introduction

Remediation of hydrocarbon-contaminated sites using microorganisms provides a cheap and environmentally sound solution for removal of oil pollutants. Bacteria capable of utilizing alkanes as carbon and energy source have drawn significant interest in the last few decades. Many bacterial species have been identified as potential hydrocarbon biodegraders (Throne-Holst et al. 2006; Feng et al. 2007; Demnerova et al. 2008; Lo Piccolo et al. 2011; Uhlik et al. 2012; Li et al. 2013).

Members of the *Rhodococcus* genus are among the most promising hydrocarbon-degrading microorganisms. Rhodococci produce various surface active agents: low molecular weight trehalolipids (White et al. 2013; Inaba et al. 2013) or high molecular weight exopolysaccharides (Urai et al. 2007a, b; Perry et al. 2007) to lower the surface tension and increase the bioavailability of water-insoluble substrates. Rhodococcal cell walls contain mycolic acids, which provide

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hydrophobic characteristics to the cell surface (Stratton et al. 1999; Kolouchová et al. 2012). This is a common feature among the mycolata group and the chain length of the mycolic acids is characteristic to the genus. Moreover, rhodococci are also able to change their membrane composition and cell surface properties in response to organic substrates (de Carvalho et al. 2005; 2009).

Besides petroleum hydrocarbons, they can also utilize xenobiotics, such as polychlorinated biphenyls (Maeda et al. 1995; Kosono et al. 1997) or antifungal agents, e.g., carbendazim (Zhang et al. 2013). Certain strains of rhodococci were shown to degrade mycotoxins: zearalenone (Kriszt et al. 2012), or aflatoxin B_1 (Alberts et al. 2006).

Rodococcus erythropolis is a well-known species among rhodococci. Numerous industrially and environmentally important enzymatic reactions have been described in various strains and have been reviewed by de Carvalho and da Fonseca (2005).

R. erythropolis PR4 was isolated from the Pacific Ocean (Komukai-Nakamura et al. 1996). In our preliminary study, it was shown that the strain could grow both on hexadecane and diesel oil (Kis et al. 2013). The genome sequence of the strain has been determined (Sekine et al. 2006.). Four predicted alkane-1-monooxygenases are encoded in the relatively large 6.5 Mbp chromosome. This strain also harbors two circular plasmids and one large linear plasmid, the latter carries the genes of two enzyme sets assumed to be involved in alkane oxidation (Sekine et al. 2006). In addition to *alkB* (alkane-1-monooxygenase) genes, numerous other oxygenase coding genes could also be identified in the genome, such as 16 cytochrome P450 (CYP), 3 multicopper oxidase (MCO) and many other mono- and dioxygenase genes. Alkane monooxygenases are thought to be responsible for the oxidation of alkanes of various length, CYPs have more diverse substrate specificity, while dioxygenases usually oxidize various aromatics (Fuentes et al. 2014).

The development of deep sequencing techniques opened up new vistas both in genomics and transcriptomics. RNA-Seq is a relatively novel technique for whole transcriptome analysis based on deep sequencing methods. RNA-Seq has many benefits over a traditional microarray, for example it is not preconceptual and it is more sensitive, thus it can detect novel transcripts. Moreover, the single base resolution of the next generation sequencing techniques provides the opportunity to discover or revise genes and determine transcript boundaries (Marioni et al. 2008; Wang et al. 2009; Fu et al. 2009; Külahoglu and Bräutigam 2014).

Metagenomic and metatranscriptomic studies of alkane or oil degrading microbial communities in soil and sea water have been reported in the last 5 years (Yergeau et al. 2009; Mason et al. 2012). Moreover, whole transcriptome analysis of *Rhodococcus jostii* RHA1 strain has been carried out using microarray technology. In these experiments, the biphenyl (Goncalves et al. 2006) and the terephthalate (Hara et al. 2007) catabolic pathways were identified. A few studies also investigated the proteomic and transcriptomic background of alkane biodegradation in *Alcanivorax borkumensis* (Sabirova et al. 2006; 2011; Naether et al. 2013), a predominant bacterium in crude oil-contaminated seawater which primarily utilizes alkanes. Hexadecane is a model compound for aliphatic hydrocarbon degradation (Sabirova et al. 2011). However, deep sequencing-based comparative transcriptome analysis of a single hydrocarbon-degrading *Rhodococcus* strain, grown on model and real industrial substrates, has not been performed.

In this study, we aimed to elucidate the complex mechanisms behind hydrocarbon biodegradation by comparative transcriptome analysis using SOLiD™ (Life Technologies Co. Carlsbad, CA, USA) next-generation sequencing system. We investigated the metabolic response of our model organism, *R. erythropolis* PR4, exposed to hexadecane, diesel oil and an artificial hydrocarbon mixture in reference to the values of acetate grown cells. The transcriptomic data obtained for cells grown on various hydrocarbons contribute to the functional characterization of this rhodococcal genome, disclose new potential components in diesel utilization and assign novel target genes for environmental monitoring projects.

Materials and methods

Strain and growth conditions

R. erythropolis PR4 (NBRC 100887) was obtained from the National Institute of Technology and Evaluation, Biological Resource Center (NBRC, Kisarazu-shi, Chiba, Japan).

The strain was maintained on Luria Broth (LB) medium and agar plates (LB: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl; agar plates contain 15 g/L agarose) and stored at 4 °C. Starter cultures were inoculated in LB medium and grown at 25 °C with shaking (160 rpm) until OD_{600}=1. Cultures were centrifuged at 15,000×*g* for 10 min at 4 °C, then the pellet was washed with fresh minimal medium (0.217 g/L KH_2PO_4, 1.46 g/L K_2HPO_4, 0.585 g/L NaCl, 0.125 g/L $MgSO_4 \cdot 7\ H_2O$, 44 mg/L $CaCl_2 \cdot 2\ H_2O$, 0.2 mg/L $ZnSO_4 \cdot 7\ H_2O$, 0.06 mg/L $MnCl_2 \cdot 4\ H_2O$, 0.6 mg/L H_3BO_3, 0.4 mg/L $CoCl_2 \cdot 6\ H_2O$, 0.02 mg/L $CuCl_2 \cdot 2\ H_2O$, 0.04 mg/L $NiCl_2 \cdot 6\ H_2O$, 0.046 mg/L $NaMoO_4 \cdot 4\ H_2O$, 14 mg/L $FeSO_4$, 9.3 mg/L EDTA·2 H_2O, 1.2 g/L NH_4NO_3)

Starter cultures (4 *v*/*v*%) were inoculated into 5 L minimal medium in a Biostat C Fermenter (B. Braun Biotech International GmbH, Melsungen, Germany). The following substrates were used as carbon and energy sources: 3 % (*m*/*v*) sodium acetate (A); 1 % (*v*/*v*) hexadecane (HeD); 1 % (*v*/*v*) diesel oil (DiO) (MOL Group Plc. Budapest, Hungary, MSZ EN 590; PAH content: <11 %; sulfur content: <10 mg/kg;

FAME content: <7 %); artificial combinations of pure hydrocarbons: hexadecane supplemented with cyclic compounds, cycloparaffins and aromatics (HeD+C) contained 1 % (*v*/*v*) hexadecane, 0.0057 % (*v*/*v*) benzene, 0.0057 % (*v*/*v*) toluene 0.0057 % (*v*/*v*) ethyl-benzene, 0.0057 % (*v*/*v*) xylene, 0.025 % (*v*/*v*) cyclohexane, 0.02 % (*m*/*v*) naphthalene, 0.02 % (*v*/*v*) tetralin and 0.022 % (*v*/*v*) decalin; while hexadecane supplemented with branched chain hydrocarbons (HeD+B) consisted of 1 % (*v*/*v*) hexadecane, 0.029 % (*v*/*v*) 2,2,4-trimethyl pentane, 0.023 % (*v*/*v*) squalane and 0.023 % (*v*/*v*) squalene; 1–1 % (*v*/*v*) light, normal and heavy diesel fractions used separately (MOL Group Plc. Budapest, Hungary). All media were also supplemented with 0.05 % (*m*/*v*) amino acid mix (0.0026 % (*m*/*v*) containing each amino acid except for glycine) to accelerate cell growth in the early phase of fermentation. According to the literature (Urai et al. 2007a, b), the temperature was kept at 25 °C, the pH was set at 7.5 and maintained with controlled addition of 1 M NaOH or HCl. Pure oxygen 4.5 (Linde Gas Hungary Co. Ltd., Budapest, Hungary) was used for aeration at a gas flow rate of 100 mL/min. Oxygen saturation was controlled with a stirring rate between 200 and 360 rpm. Initial stirring velocity was set to 100 rpm in the case of cultures grown on sodium acetate. Cultures grown on sodium acetate were used as controls. Each fermentation was performed in three replicates.

Biodegradation experiments using diesel oil fractions (1–1 % (*v*/*v*) light, normal and heavy fractions obtained from MOL Group Plc. Budapest, Hungary) were performed separately.

GC/MS analysis

The hydrocarbon content of the samples was extracted with 1/3 volume of *n*-hexane or diethyl-ether. Samples were diluted tenfold, then injected to an Agilent 6890 gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA), equipped with a flame ionization detector (FID) and a HP-5MS (30 m× 25 mm×25 μm) capillary column. Samples were split between FID and Agilent 5975C VL MSD (Agilent Technologies Inc., Santa Clara, CA, USA) in a ratio of 1:1. Compounds were identified by means of the NIST/EPA/NIH Mass Spectral Library with Search Program (http://www.nist.gov/srd/nist1a.cfm). In the case of samples with defined compositions, calibration curves were generated for each compound for quantitative evaluation of substrate consumption.

Total nucleic acid purification

Three milliliters of bacterium culture was centrifuged at 12,000×*g* for 2 minutes at room temperature. Hydrocarbons were extracted from the samples by liquid-liquid extraction with 2/3 volume of chloroform prior to centrifugation. Cells were disrupted with acid purified sea sand (Fluka Chemie AG, Buchs, Switzerland) and resuspended in RLT Buffer (Qiagen RNeasy Mini Kit). After centrifugation, the supernatant was mixed with an equal volume of ice cold 70 % (*v*/*v*) ethanol. Samples were loaded onto the Qiagen RNeasy (Qiagen Inc., Valencia, CA, USA) binding column. Subsequent washing steps and elution were carried out according to the manufacturer's instructions. Nucleic acid concentrations were measured with NanoDrop 1000 (Thermo Fischer Scientific Inc, Waltham, USA).

RNA purification

Preliminary experiments were performed in order to establish the growth phases of the cells cultivated on various substrates. Samples for RNA preparation were taken in the middle-late exponential phases: in the 21st hour in the case of cultures grown on diesel oil and hexadecane, in the 31st hour from cultures grown on sodium acetate and in the 23rd hour in the case of artificial hydrocarbon mixtures. RNA purification was performed according to the protocol developed for actinomycetes (Nagy et al. 1997) with slight modifications. Briefly, 12 mL bacterium culture was centrifuged at 15,000×*g* for 5 min at 4 °C. The cell pellet was frozen in liquid nitrogen then disrupted with acid purified sea sand (Fluka Chemie AG, Buchs, Switzerland). RNA samples were purified with RNeasy™ Plus Mini Kit (Qiagen Inc., Valencia, CA, USA). RNase free DNase I (Invitrogen, Carlsbad, CA, USA) was used for digestion of residual DNA in the samples.

Whole transcriptome analysis

Whole transcriptome analysis was carried out using a SOLiD 5500XL™ next generation sequencer (Life Technologies Co. Carlsbad, CA, USA). RNA from the three biological replicates was pooled and ribosomal RNA was removed using the Ribo-Zero™ rRNA Removal Kit for Gram-Positive Bacteria (Epicentre Biotechnologies, Madison, WI, USA). Library preparation and RNA sequencing were performed by using the dedicated SOLiD 5500XL kits (Life Technologies). Approximately 20–25 million 50-nucleotide-long reads were generated per sample, out of these, 45–60 % proved to be of high quality and could be mapped onto the *R. erythropolis* PR4 genome.

Mapping of reads to the reference genome, normalization and calculation of expression values were performed using the CLC Genomic Workbench software (CLC Bio A/S, Aarhus, Denmark). Reads mapping to tRNA and rRNA were removed from further analysis. In order to identify up- and downregulated genes, RPKM values were compared. BLASTX(P) (http://blast.ncbi.nlm.nih.gov) searches were executed in Swissprot/Uniprot (http://www.uniprot.org/) and the non-redundant databases for functional assignment of the putative

genes of interest. Conserved domains were predicted by the NCBI Conserved Domain database and the SMART program (http://smart.embl-heidelberg.de/).

cDNA synthesis and RT-qPCR

The DNAse I treated RNA samples (1 μg) were reverse transcribed into cDNA with Maxima First Strand cDNA Synthesis Kit (Thermo Fischer Scientific, Inc. Waltham, USA). The cDNA products were diluted fivefold and RT-qPCR was performed with Power SYBR® Green Master Mix (Life Technologies Co. Carlsbad, CA, USA) in ABI 7500 Real Time PCR System (Life Technologies Co.). After 2 min at 50 °C and 10 min denaturation at 95 °C, the following cycles were performed 40 times: 15 s at 95 °C and 1 min at 60 °C. Primers were used in a final concentration of 0.5 pmol/μL. Primer sequences are shown in Supplementary materials, Table S1.

Cycle threshold (C_t) values were determined by automated threshold option with ABI Sequence Detection Software v1.4. (Life Technologies Co.) The C_t values of the samples were normalized (ΔC_t) with C_t values of 16S rRNA. Expression fold change was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) where $\Delta\Delta C_t$ was obtained by subtracting ΔC_t of the reference samples from ΔC_t of the samples of interest.

Phylogenetic analysis

The phylogenetic trees of CYP and AlkB sequences were produced according to the strategy described earlier (Fülöp et al. 2012). Briefly, T-COFFEE (Notredame et al. 2000) was used for multiple alignment, PhyML (http://www.atgc-montpellier.fr/phyml/) (Guindon et al. 2010) was applied for construction of the phylogenetic trees visualized by the Figtree v1.4.0. software (http://tree.bio.ed.ac.uk/software/figtree/).

Availability of supporting data

The NGS based transcriptomic data were deposited with the NCBI GEO accession number: GSE56474 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56474)

Results

R. erythropolis PR4 can catabolize various hydrocarbons

Although it has been recently demonstrated that the *R. erythropolis* PR4 strain could utilize both hexadecane and diesel fuel as nutrients, the substrate specificity of the cells was not analyzed. Mass spectrometric monitoring of the biodegradation of various diesel oil fractions indicated that the strain had broad substrate specificity (Supplementary materials, Fig. S1) and could simultaneously convert various hydrocarbons including aliphatic and aromatic compounds. Therefore, it should have a complex apparatus to catabolize various types of hydrocarbons, including aliphatic, cyclic, saturated, non-saturated and substituted mono- and polyaromatic compounds. However, it is not clear which gene products are involved in the biodegradation of simple or complex hydrocarbon substrates. Moreover, hydrocarbons can influence the expression profile of a number of metabolic routes, including fatty acid biosynthesis, biosurfactant biosynthesis, etc. In the next sections, we focused on the comparative transcriptome analysis of cells grown on the model compound hexadecane (HeD), on commercial diesel oil (DiO) and on artificial hydrocarbon mixtures. For reference, we have tried several substrates: glucose, sugars, organic acids and complex media. Among these nutrients, acetate seemed to be the best choice. The growth curve of cells cultivated on acetate had a longer lag phase but the growth period had similar kinetics as compared to that of cells grown on hydrocarbons (Fig. 1). Therefore, acetate was chosen as reference nutrient.

Fermentation and cell growth

R. erythropolis PR4 was cultivated in minimal medium, containing hexadecane, diesel oil, or sodium acetate as carbon and energy sources (for details see "Materials and methods"). Total nucleic acid content of the cultures was used to characterize cell growth. The exponential phase of the growth on hydrocarbons started after 18 h, while cultures grown on sodium acetate had longer lag phases and exponential growth started after the 28th hour (Fig. 1). The oxygen concentrations in the fermenters were continuously monitored and decreases in oxygen saturation of the culture medium were in concordance with cell growth (data not shown).

Samples were taken at the points indicated in Fig. 1, which corresponded to the middle-late exponential growth phase. In this growth phase, every culture was homogeneous with no detectable biofilm formation, either on the top of the medium or on the glass.

Sequencing results

Total RNA was purified from three pooled, parallel samples, taken at the points indicated in Fig. 1. The RNA was processed according to the protocols described in the "Materials and methods" section. Finally, whole transcriptome analyses were performed for all samples using a SOLiD™ sequencing platform. The reads were mapped onto the genome, their distribution analyses were carried out (see "Materials and methods") and the *R*eads *P*er *K*ilobase of gene model per *M*illion mapped reads (RPKM) values were compared. Three comparisons were made: hexadecane vs. acetate, diesel

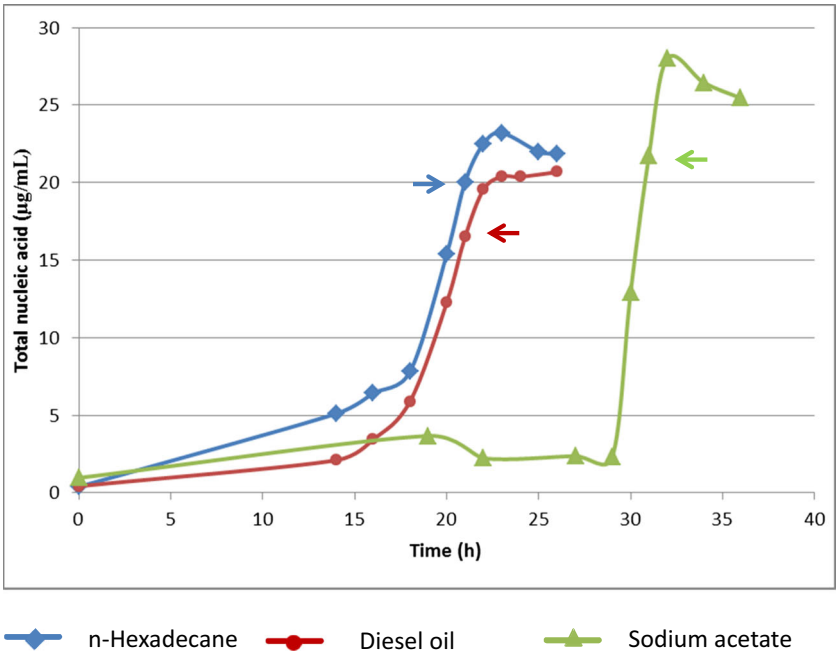

Fig. 1 Fermentation growth curves of *R. erythropolis* PR4 cultivation on sodium acetate, *n*-hexadecane and diesel oil. Time points of sampling are indicated by *arrows*

vs. acetate and diesel vs. hexadecane. In the Supplementary materials, Online Table S2 lists almost 200 annotated ORFs which exhibited more than three times variation in their expression. They were aggregated into gene groups according to their functions, such as hydrocarbon oxidation, acyl-CoA synthesis, β-oxidation pathway, fatty acid and mycolic acid synthesis, iron transport and siderophore synthesis, exopolysaccharide synthesis, etc. Online Table S2 contains the RPKM values of samples grown on acetate and the transcriptional *-fold changes* of the sample pairs indicated.

In addition to Online Table S2, Fig. 2a illustrates the most prominent changes in the transcriptional level of *alkB*, *cyp* and several other oxygenase genes.

Twenty-four genes were selected for validation by RT-qPCR (Table 1, Fig. 2b). Out of these, expression values of 19 genes were in semi-quantitative concordance with transcriptomic data. However, the changes in transcript levels of 5 genes did not coincide with the transcriptomic data. All the genes with inconsistent expression data had low RPKM values (<1.5), both on acetate and hexadecane in the whole transcriptome analysis, therefore all genes having such low RPKM values were omitted from further analysis and interpretation. In the following sections, the data obtained from various relevant functional groups are discussed.

Hydrocarbon oxidation

The first step in biodegradation of *n*-alkanes is oxidation into their corresponding alcohol. Four alkane monooxygenase genes were identified in the genome of *R. erythropolis* PR4. They might differ in their substrate specificity by preferring alkanes of distinct length. The comparison of the expression of the *alkB* genes disclosed that only two *alkB* genes were highly upregulated by both hexadecane (HeD) and diesel oil (DiO) (Fig. 2a–b, Table 1). The induction level of RER_07460 (*alkB1*) was much higher when compared to that of RER_21620 (*alkB2*). On the other hand, *alkB1* had abated expression in the presence of DiO in comparison to HeD. The mRNA level of the other two *alkB* genes, RER_24030 and RER_54580 did not change significantly. A phylogenetic analysis revealed that RER_24030 and RER_54580 are separated from the other two inducible genes, suggesting that RER_24030 and RER_54580 ORFs were transferred to *R. erythropolis* via horizontal gene transfer (data not shown). This idea was supported by the fact that neither RER_24030 nor RER_54580 had adjacent typical genes coding for redox partner proteins (rubredoxin and its reductase, see below). The expression levels of all *alkB* genes were low (compared to that of other genes) in cells grown on acetate (Online Table S2). From these data, AlkB1 seems to be the dominant alkane-1-monooxygenase, but AlkB2 likely has an additional important role.

In the genome, both *alkB1* (RER_07460) and *alkB2* (RER_21620) genes are followed by two rubredoxin genes: RER_07470, RER_07480, as well as RER_21630 and RER_21640. The *alkB1* locus contains a rubredoxin reductase gene (RER_07490) as well. The *alkB* type monooxygenases are usually associated with rubredoxins, which are oxidized during the alkane monooxygenation process. The transcription of RER_07470 rubredoxin gene was 1120-fold higher in cells cultivated on HeD relative to the values obtained for cultures propagated on acetate and 272-fold upregulation could be observed in cells grown on DiO in reference to the acetate based samples (Online Table S2). Rubredoxin

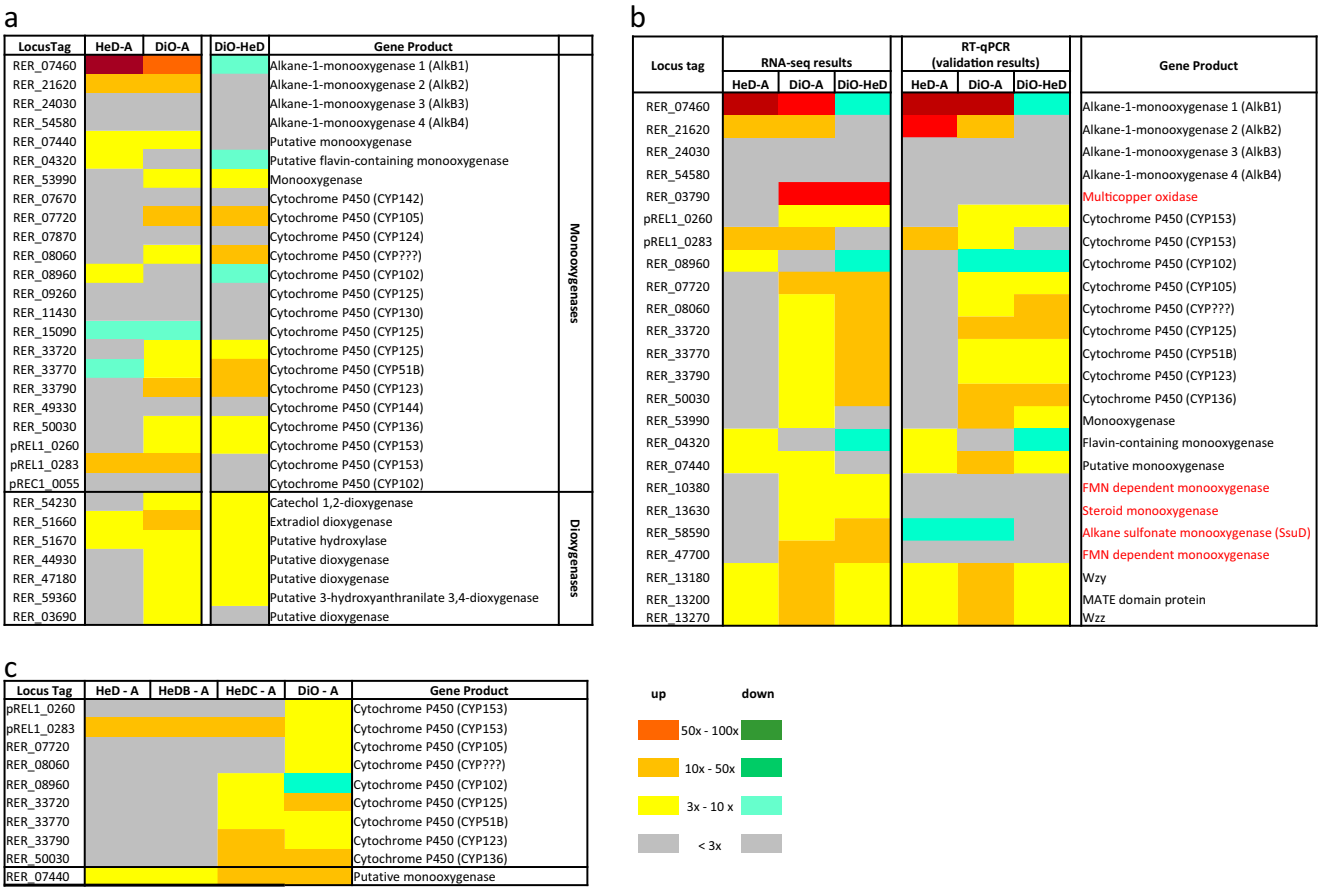

Fig. 2 Heat map of the relative transcript levels of the most prominent oxygenase and a few other genes. **a** Whole cell transcriptome analysis (WTA), **b** Comparative representation of the expression levels of selected genes measured by WTA and RT-qPCR, **c** Transcript level of *cyp* and monooxygenase genes in cells grown on various artificial mixtures (RT-qPCR). *HeD-A column* Hexadecane vs. acetate, *DiO-A column* Diesel oil vs acetate; *DiO-HeD column* Diesel oil vs hexadecane, *HeDB-A column* hexadecane+branched alkanes vs acetate, *HeDC-A column* hexadecane+cycloparaffins+aromatics vs acetate comparisons. The color codes are shown in the figure, up and down mean upregulation and downregulation, respectively

reductases are responsible for re-reduction of rubredoxins using NAD(P)H (Hagelueken et al. 2007). The rubredoxin reductase gene, RER_07490, was also highly upregulated (more than 100-fold change) in samples grown on either hydrocarbon substrates, relative to the values obtained for acetate.

Downstream to the *alkB1*-related rubredoxin reductase gene (RER_07490), three consecutive ORFs: RER_07500, RER_07510, RER_07520 had massively elevated expression rates in HeD grown cells (HeD vs Ac: 76–146-fold increase) (Online Table S2). RER_07500 is a putative TetR family regulator. Members of this regulator family are known as transcriptional repressors, regulating themselves and the adjacent operon (Ahn et al. 2012). Therefore, their elevated mRNA level upon induction coincides well with their regulatory mechanism. The relative genomic location of the gene coding for TetR-like protein (in proximity of the *alkB* operons) is a conserved feature among rhodococci and other actinobacteria. However, the expression of the other *tetR*-like gene, RER_21650, adjacent to the *alkB2* operon, did not change significantly on either carbon source.

Downstream to the *alkB1* operon, the two other subsequent ORFs are RER_07510 and RER_07520. Both genes are massively induced: 106- and 146-fold upregulation on HeD and 29- and 24-fold increase on DiO grown samples relative to the control (Online Table S2). The gene product of RER_07510 is a 56-amino acid long oligopeptide chain, while RER_07520 encodes a 277 amino acid long protein. BLAST search for RER_07510 could not identify this ORF in the recently published *R. erythropolis* CCM2595 genome (Strnad et al. 2014). In contrast, RER_07520 gene product resembled hypothetical proteins from other *Rhodococcus* species. A SMART analysis revealed a 29-amino acid signal peptide on the N-terminal region of the protein, which suggested that this protein was secreted. According to their genomic context, interactions were predicted by the STRING tool (http://string-db.org/) between these two ORFs and the rubredoxin and *alkB1* genes. Their functions, however, remain elusive.

Table 1 Expression changes of selected genes in cultures grown on various hydrocarbons measured by RT-qPCR. *HeD-A*: Hexadecane vs. acetate; *HeDB-A*: hexadecane+branched alkanes vs acetate; *HeDC-A*: hexadecane+cycloparaffins+aromatics vs acetate; *DiO-A*: Diesel oil vs acetate comparisons

Locus tag	HeD-A expression fold change	HeDB-A expression fold change	HeDC-A expression fold change	DiO-A expression fold change	Product
RER_07460	2271.3±79.8	1232.8±292.9	1558.2±147.1	601.9±12.9	Alkane-1-monooxygenase 1
RER_21620	53.1±7.0	81.2±17.0	97.8±26.6	37.5±3.3	Alkane-1-monooxygenase 2
RER_24030	−2.5±0.1	Not analyzed	Not analyzed	−2.6±0.2	Alkane-1-monooxygenase 3
RER_54580	1.5±0.3	Not analyzed	Not analyzed	1.9±0.2	Alkane-1-monooxygenase 4
RER_03790	1.8±0.6	1.3±0.2	−1.3±0.1	2.9±0.9	Multicopper oxidase
pREL1_0260	1.9±0.2	1.5±0.1	1.5±0.06	4.0±0.4	CYP 153_1
pREL1_0283	11.2±1.4	12.1±0.7	14.3±0.3	8.1±0.2	CYP 153_2
RER_08960	2.0±0.2	2.5±0.1	3.9±0.6	−3.1±0.3	CYP 102
RER_07720	1.5±0.4	1.3±0.2	1.9±0.08	6.9±0.3	CYP 105
RER_08060	−1.1±0.9	−1.3±0.2	2.2±0.3	8.2±2.5	CYP ???
RER_33720	−2.0±0.1	1.7±0.5	6.2±0.5	10.0±0.4	CYP125
RER_33770	−2.1±0.1	1.2±0.3	5.5±0.6	3.7±0.3	CYP51B
RER_33790	−2.0±0.1	1.9±0.4	11.2±1.0	6.5±0.3	CYP123
RER_50030	−1.1±0.06	1.0±0.08	20.3±0.05	30.4±1.6	CYP136
RER_53990	2.8±0.2	Not analyzed	Not analyzed	14.8±0.3	Monooxygenase
RER_04320	11.2±0.9	Not analyzed	Not analyzed	1.9±0.3	Flavin-containing monooxygenase
RER_07440	3.4±0.5	4.1±0.4	15.0±1.3	25.4±0.7	Putative monooxygenase
RER_10380	1.1±0.08	Not analyzed	Not analyzed	−1.3±0.09	FMN dependent monooxygenase
RER_13630	−1.3±0.2	Not analyzed	Not analyzed	−2.8±0.5	Steroid monooxygenase
RER_58590	−5.3±1.3	Not analyzed	Not analyzed	−3.2±0.3	SsuD (Alkane sulfonate monooxygenase)
RER_47700	−2.1±0.1	Not analyzed	Not analyzed	−2.4±0.2	FMN dependent monooxygenase
RER_13180	4.7±0.2	Not analyzed	Not analyzed	43.9±3.0	Wzy
RER_13200	8.0±0.09	12.7±0.8	90.4±10.6	72.6±1.1	MATE domain protein
RER_13270	4.8±1.4	Not analyzed	Not analyzed	18.2±0.8	Wzz

We found another gene cluster, RER_04310–04340, induced by HeD (8–12× increase), but not by DiO. A TetR family transcriptional regulator, a flavin-containing monooxygenase, an oxidoreductase and a hypothetical protein are encoded in this region, in this order. These enzymes can provide an alternate oxidation pathway; however, the actual role of these gene products in the biodegradation of hexadecane is still unknown.

The cytochrome P450 (CYP) superfamily is a large group of iron-heme proteins, commonly found in prokaryotes and eukaryotes. In total, 16 *cyp* genes were identified in the genetic material of *R. erythropolis* PR4: 13 genes in the chromosome, 2 genes in the large linear plasmid and 1 in the first circular plasmid. The transcript levels of the *cyp* genes located in the large linear plasmid are in the middle range in cells grown on acetate containing media: they have 30–60× higher transcript level than the *alkB1* and *alkB2* genes (Online Table S2). Among these, only the expression of pREL1_0283 (located on the linear plasmid) was significantly elevated under both hydrocarbon assimilating conditions (Fig. 2a, Table 1). The gene encodes a cytochrome P450 enzyme belonging to CYP153 family. In cells grown on acetate, the expression levels of genomic *cyp* genes were similar to those of *alkB* genes. Based on WTA and RT-qPCR results, the expression of eight *cyp* genes, including pREL1_0283, was upregulated in samples cultivated on DiO (Fig. 2a–c, Table 1). The extent of induction was between 4 and 30 times, measured by RT-qPCR analysis. The deduced gene products were classified according to the literature (McKinnon et al. 2008) and a phylogenetic tree was constructed (see "Materials and methods"). Their analysis showed that (a) the inducible genes were located on diverse branches and classes of the tree (Fig. 3); (b) in addition to the enzymes of the CYP153 family, other types of cytochrome P450 (e.g. members of CYP105 and CYP123 families) might be involved in hydrocarbon degradation. This is plausible, since diesel fuel having diverse composition should be oxidized by diverse enzymes.

In order to identify diesel component groups responsible for the upregulation of *cyp* genes and a putative monooxygenase gene (RER 07440), we performed additional fermentations on hexadecane (HeD); hexadecane

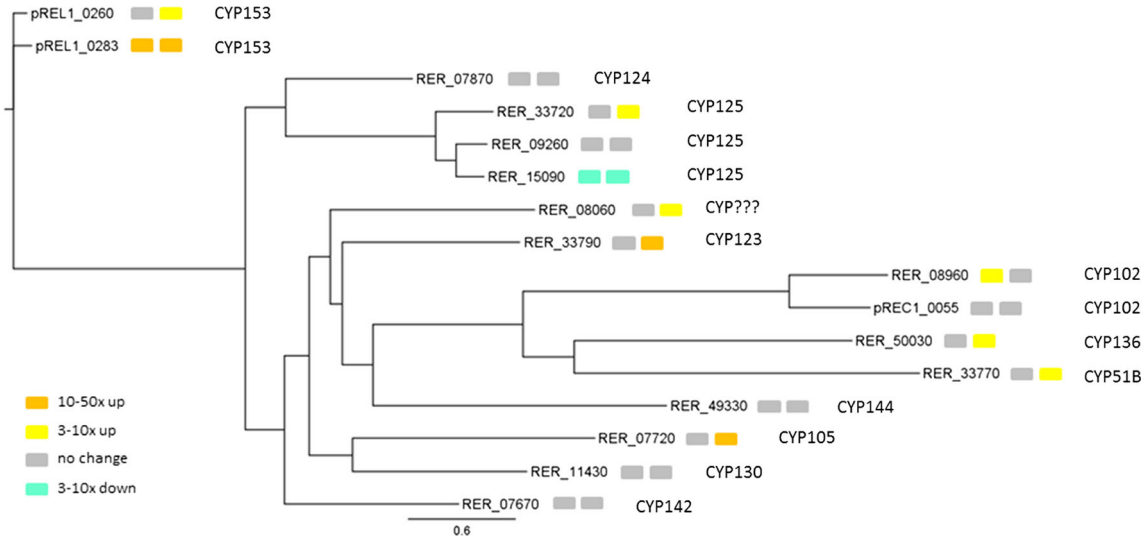

Fig. 3 Phylogenetic tree of the CYP proteins encoded in the genome of *R. erythropolis* PR4. *Colored boxes* have the same code as in Fig. 2 and are illustrated on the figure. In each case, the *first box* of every branch represents hexadecane vs acetate, the *second box* shows the transcriptional changes in diesel oil vs acetate grown cells. CYPxxx means the cytochrome P450 class the given gene product belongs to. The classification RER_08060 is uncertain, might be a new type of P450

supplemented with aromatics and cycloparaffins (HeD+C) and hexadecane supplemented with branched chain hydrocarbons (HeD+B). GC/MS analysis revealed that the various components of the mixtures were simultaneously converted (data not shown). It suggests that the cells do not have apparent priority ranking for the hydrophobic substrates/compounds. RNA samples were taken in the middle-late logarithmic phase of the cell growth and RT-qPCR measurements were carried out. Six of the nine examined *cyp* genes were upregulated by HeD+C by a factor of 3.9 to 20 (Table 1, Fig. 2c). Only pREL1_0283 (Cyp153) and the putative monooxygenase (RER07440) were induced in the presence of all hydrocarbon nutrients. RER_33720, RER_33770, RER_33790 and RER_50030 have high expression values both on HeD+C and DiO. The gene of a putative monooxygenase (RER07440) had slightly elevated expression in the presence of non-cyclic hydrocarbons, but its transcription was strongly upregulated in cells grown on both HeD+C and DiO (Table 1). Thus, the products of these genes seem to have a role in the oxidation of cyclic hydrocarbons. RER_08960 coding for CYP102 had slightly increased expression levels on HeD+C, but its transcript level is downregulated in cells grown on DiO. These changes were very close to the threshold value: 3× up- or downregulation. Three other *cyp* genes were only upregulated in the presence of DiO, which suggests they have alternative roles.

The genomic contexts of *cyp* genes are quite heterogeneous. The genes are flanked by fatty acid-CoA ligase, aldehyde dehydrogenase and TetR-type regulator coding genes. However, on the linear plasmid, the *cyp* genes are flanked by ferredoxin (pREL1_0259 and pREL1_0282) and ferredoxin reductase (pREL1_0261 and pREL1_0284) genes. These ferredoxin and ferredoxin reductase genes followed the expression pattern of the corresponding cytochrome P450 genes. Nevertheless, the linkage between the various CYP proteins, ferredoxins and ferredoxin reductases is still to be established.

Further examination of the genomic context of pREL1_0260 reveals three other genes which are presumably involved in the downstream reactions of alkane oxidation. The first gene, pREL1_0257, codes for a zinc-containing alcohol dehydrogenase responsible for the conversion of alcohols to aldehydes, while pREL1_0258 encodes an aldehyde dehydrogenase which catalyzes the oxidation of aldehydes to the corresponding fatty acid. A fatty acid-CoA ligase encoded by pREL1_0256 activates the fatty acids by attaching them to coenzyme A. Surprisingly, all three genes were induced by only DiO; their expression in the presence of HeD was not remarkably elevated as compared to the control.

Figure 2a shows that the transcription of seven dioxygenase genes was upregulated in the presence of DiO. From these, the expression level of only one gene (RER_51660) was induced more than 10 times in cells cultivated in DiO. Involvement of HeD in the medium slightly elevated the transcript level of only this gene (3.1-fold increase relative to the control (Fig. 2a and Online Table S2)).

RER_51660 encodes an extradiol dioxygenase, which likely catalyzes aromatic ring cleavage. Its transcription was increased by a factor of 12 in cells grown on DiO. In the genome, it is followed by a hydroxylase gene RER_51670, which is also upregulated by DiO, while the transcript level of RER_51680, annotated as hydrolase, was practically the same in all samples studied. Therefore, the gene products of RER_51670 and RER_51660 might be necessary for ring hydroxylation and cleavage of aromatic/benzene compounds in DiO.

Fatty acid metabolism

The final products of *n*-alkane oxidation are CoA-activated fatty acids, which are utilized in the β-oxidation process, or assimilated as membrane components, or used as precursors for mycolic acid synthesis.

We observed that alkanes induced the expression of genes encoding enzymes involved in the β-oxidation of fatty acids (Online Table S2). In the first step of the oxidation cascade, an acyl-CoA dehydrogenase oxidizes the beta carbon of the fatty acid yielding 2,3-*trans*-enoyl-CoA. Numerous acyl-CoA dehydrogenase genes were identified and two of them had relatively high transcript levels even in acetate grown cells (RER_11300, RER_50060). Their expression was further increased by factors between 5 and 8 in the presence of either HeD or DiO (Online Table S2). The expression of a few acyl-CoA dehydrogenase genes (RER_52580, RER_27750 and RER_50190), having much lower transcript levels, could be induced by alkanes. RER_52580 (*fadE*) showed the greatest increase in expression on HeD; however, we observed two-fold lower expression levels in cells grown on DiO as compared to HeD. Several acyl-CoA dehydrogenase genes had elevated expression levels only in DiO cultivated cultures (e.g., RER_50750: 13-fold, RER_50770: 18-fold, RER_57810: 11-fold increase compared to the control) (Online Table S2). In spite of the selective hydrocarbon sensitivity of the expression of these genes, their basic transcript levels were low. Therefore, RER_11300 and RER_50060 seem to be the most important acyl-CoA dehydrogenase genes; their expressions are hydrocarbon inducible.

An operon composed of two ORFs (RER_28420 and RER_28430) had relatively high basic expression, which was slightly induced by alkanes. RER_28420 encodes a FadJ-like protein which possessed both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity, while the product of RER_28430 is a 3-ketoacyl-CoA thiolase (FadA), which catalyzes the final step of the β-oxidation route. There are other hydrocarbon responsive enoyl-CoA hydratase genes, but their expression levels are rather low. Thus, the genes of the β-oxidation pathways were moderately upregulated in the presence of hydrocarbons.

In the majority of mycolic acid containing actinomycetes, two types of fatty acid synthase (FAS) systems coexist. The eukaryotic-type FAS I consists of one large polypeptide chain carrying all domains involved in fatty acid chain elongation. *R. erythropolis* PR4 FAS I shares 63–65 % identity in amino acid sequence with its mycobacterial homologous counterparts. We observed a remarkable HeD-dependent downregulation of fatty acid synthase I (RER_38730, 140-fold decrease) and the adjacent acyl carrier protein transacylase (RER_38720, 31-fold drop) (Online Table S2). In contrast, the expression of FAS I was 30-fold higher in cells grown on DiO as compared to HeD, but it was still downregulated relative to the control. We assume this difference might be owing to the lower relative concentration of mid-chain alkanes in DiO. The fatty acids derived from alkanes of proper length might not be enough for the cellular requirements; therefore, in the case of cells grown on DiO, the de novo biosynthesis of fatty acids should be more active as compared to those grown on HeD.

FAS II is a prokaryotic-type multienzyme system of fatty acid synthesis. In contrast to FAS I, neither of the genes of the FAS II system, nor the polyketide synthase 13 homologue (RER_02210), was up- or downregulated by hydrocarbons, which can be explained by the fact that mycolic acids are essential cell wall components in rhodococci and related genera in various media.

Exopolysaccharide production

A 15-kb-long region between RER_13170 and RER_13290, except for RER_13280, was upregulated by hydrocarbons (Online Table S2). The transcript levels of RER 13180, 13200 and 13270 were measured by RT-qPCR, as well (Fig. 2b, Table 1). A much more prominent induction could be observed in cells cultivated on DiO (18–73× increase vs acetate) as compared to the cell grown on HeD (3–8× increase vs acetate) (Online Table 1, Table S2). The RER_13170, RER_13190, RER_13210, RER_13220 and RER_13230 encode various glycosyltransferases, catalyzing the transfer of sugar molecules from the activated donor to the acceptor molecule. Other activated sugar residue modifying enzymes are also encoded in this genomic region: two UDP-glucose 6-dehydrogenases (RER_13260 and RER_13290), one GDP-L-fructose synthase (RER_13250) and one GDP-mannose 4, 6-dehydrogenase (RER_13240). A transmembrane protein, containing a WzyC-like domain, is encoded by RER_13180, while a Wzz domain is present in the gene product of RER_13270. Wzy is responsible for the elongation of O-antigen and Wzz determines the chain length of the exopolysaccharide in *Escherichia coli* (Whitfield 2006). The gene product of RER_13200 is a 403 amino acid long hypothetical membrane protein containing 11 transmembrane helices. According to the NCBI Conserved Domain database, this protein belongs to the multidrug and toxic compound extrusion (MATE) domain containing proteins. MATE proteins use electrochemical gradients to transfer molecules across the cell membrane (Omote et al. 2006).

Based on the blastp results and SMART domain prediction, we suggest that the upregulated genes are involved in exopolysaccharide biosynthesis. These macromolecules provide matrix for the cells and thereby protection from toxic compounds. Certain exolipopolysaccharides are able to emulsify hydrophobic substrates. The expression levels of the upregulated genes involved in exopolysaccharide biosynthesis were 4–11× higher in the presence of DiO than on HeD

(Online Table S2). We assume that this intensive gene expression is caused by the toxic characteristics of the diesel oil components. RT-qPCR analyses of the expression of the MATE gene in cells grown on A, HeD, HeD+B, HeD+C and DiO further supported this idea (Table 1). The gene was significantly upregulated on all hydrocarbon carbon sources as compared to the acetate control. However, it had nine times higher expression level in cells cultivated on DiO than on HeD. These results suggest that *R. erythropolis* PR4 defends itself against hydrocarbons and, more importantly, the toxic components of DiO by producing exopolysaccharides.

Iron transport and siderophore synthesis

The expression of multiple sets of ABC transporter genes were induced by hydrocarbons (Online Table S2). The genomic region between RER_01100–RER_01130 encodes a full set of ABC transporter subunits and a major facilitator superfamily (MFS) transporter. Domain comparisons and the results of similarity searches suggest that the ABC transporter is a hydroxamate siderophore import system involved in iron transport. On the other hand, the MFS transporter encoded by RER_01100 shares 25 % identity and 41 % similarity with Ents enterobactin exporter of *Salmonella enterica* (WP_001081651), which is responsible for siderophore secretion.

Other gene clusters, RER_43410–RER_43440 and RER_57340–RER_57370, were also induced by hydrocarbons to various extents (3–24× upregulation). These regions contain genes for ABC transporters and putative siderophore-interacting proteins.

Hydrocarbon-induced, weak upregulation of a 17-kb-long gene cluster (between RER_27010 and RER_27050) was also observed. This region encodes a non-ribosomal peptide synthase (RER_27010) and proteins involved in siderophore transport.

Discussion

Several studies were presented which aimed to disclose the metabolic transcriptional response of a microbial community to oil contamination (Yergeau et al. 2009; Mason et al. 2012) or a single cell to a special type of hydrocarbon (Goncalves et al. 2006; Hara et al. 2007; Sabirova et al. 2011; Naether et al. 2013). In the papers of Goncavales et al. (2006) and Hara et al. (2007), the biphenyl and the terephthalate degradation pathways of *Rhodococcus* sp. strain RHA1 were studied, while Sabirova et al. (2011) and Neather et al. (2013) focused on the transcriptomic profiles of *A. borkumensis* grown on linear alkanes, preferring the range from C_{14} to C_{19}. *A. borkumensis* is considered as the main oil degrader, but it utilizes predominantly *n*-alkanes; therefore, it might not be able to degrade mixtures composed of various types of hydrocarbons. In this study, we presented a comparative transcriptome analysis, via high-throughput deep sequencing methods in a single bacterial strain grown on model hydrocarbons and real industrial oil. The strain was shown to be able to degrade linear, branched, cyclic and aromatic hydrocarbons simultaneously. Transcript profiles of *R. erythropolis* PR4 cells grown on HeD, DiO and sodium acetate were compared in order to get deeper insight into the metabolic responses of the cells to a model and real industrial substrates. We observed elevated expression levels of numerous genomic and plasmid-related gene clusters involved in hydrocarbon assimilation. Obviously, the expression patterns of these genes were different in samples grown on the two hydrocarbon substrates. Membrane-bound alkane monooxygenases (AlkB) catalyze this reaction by conversion of alkanes into primary alcohols by the incorporation of one half of the oxygen molecule into the alkanes while the other part is reduced by rubredoxin and a water molecule is formed (van Beilen and Funhoff 2007). Four alkane monooxygenase genes were identified in the genome of *R. erythropolis* PR4. The expression of two *alkB* genes was upregulated by both types of hydrocarbons. In *A. borkumensis*, only two *alkB* operons were identified and both of them were induced by hexadecane (Sabirova et al. 2011). Besides these genes, two flavin-containing monooxygenase genes were also upregulated. In *R. erythropolis* PR4 cells, upregulation of a flavin-containing and a putative monooxygenase gene was also observed. The relative genomic location of the gene coding for TetR-like protein (in proximity of the *alkB* operons) is a conserved feature among rhodococci and other actinobacteria. The regulation of hydrocarbon-degrading genes by TetR-type transcriptional factor is predicted on the basis of genomic context in *Rhodococcus* sp. (Cappelletti et al. 2011) and transcript analysis in *Dietzia* sp. (Procopio et al. 2013), as well. Our results serve as novel, additional, indirect proof for the role of this TetR-type protein in the transcriptional control of genes related to hydrocarbon catabolism. Direct experimental evidence is necessary to validate this idea.

Cytochrome P450 monooxygenases are alternative biocatalysts for the oxidation of aliphatic and aromatic hydrocarbons in many hydrocarbon-degrading microorganisms (van Beilen et al. 2006; Syed et al. 2013). Altogether, 16 *cyp* genes were found in *R. erythropolis* PR4. The expression of one *cyp* gene was induced by hexadecane, while elevated transcript levels were measured in the case of seven *cyp* genes in samples grown on DiO. The most dominant one belonged to the CYP153 family, the others were members of diverse CYP families. The members of CYP153 family are known to be responsible for alkane terminal hydroxylation reactions (Strnad et al. 2014). Seven *R. erythropolis* strains were described to contain two CYP153 cytochromes (Fuentes et al. 2014) and only 24 *Actinobacteria* species contained a *cyp153*

gene in addition to *alkB* genes (Nie et al. 2014a). It was recently published that CYP153 proteins can be responsible for hydroxylation of C_6–C_{10} alkanes (Nie et al. 2014b). It might be true for the gene of CYP153 (pREL1_0260), which had elevated expression in cells grown on DiO, but the transcription of CYP153 (pREL1_0283) was strongly induced by HeD, indicating its role in the hydoxylation of C_{16} hydrocarbons. Our study revealed the potential role of other types of cytochrome P450 in the biodegradation of hydrocarbons, such as CYP51B, CYP105, CYP123, CYP125 and CYP136. CYP51B was described as a sterol-demethylase (Bellamine et al. 1999), CYP105 seems to have diverse functions in catabolism of xenobiotics (Moody and Loveridge 2014.), while CYP125 was found to catalyze the terminal hydroxylation of steroids (Capyk et al. 2009). Diesel oil contains various aliphatic and aromatic hydrocarbons. Surprisingly, quite few genes coding for hydroxylase or ring-cleaving dioxygenase were upregulated by DiO and the induction levels varied between 3 and 12. Instead, CYP genes had elevated transcript levels in cells grown on diesel oil. Moreover, cyclic hydrocarbons (cycloparaffins and aromatic compounds) induced the expression of five *cyp* genes, encoding enzymes likely responsible for the oxidation of these compounds.

Ferredoxin and ferredoxin reductase are involved in the NAD(P)H-dependent electron transport associated with class I cytochrome P450 enzymes (Werck-Reichhart and Feyereisen 2000). In two cases, their genes followed the expression pattern of the corresponding cytochrome P450 genes. Nevertheless, the linkage between the various CYP proteins ferredoxins and ferredoxin reductases is still to be established. It is notable that only three *cyp* genes were identified in *A. borkumensis*, but none of them were upregulated by hexadecane (Sabirova et al. 2011), therefore there are apparent differences in the mechanism of hydrocarbon assimilation in these cells.

As was expected (Sabirova et al. 2011), components of beta oxidation were also induced by alkanes in *R. erythropolis* PR4 as well, since this pathway is responsible for the further biodegradation of fatty acids derived from alkane oxidation.

Sabirova and co-workers observed hexadecane-induced downregulation of the acetyl-CoA carboxylase enzymes in *A. borkumensis* (Sabirova et al. 2006). These enzymes catalyze the formation of malonyl-CoA from acetyl-CoA, which is the first step of fatty acid biosynthesis. The authors explained the downregulation of these enzymes by the increased exploitation of the glyoxylate bypass of the TCA cycle, which yields sufficient amounts of malate in the alkane grown cells. In contrast to their findings, HeD had no effect on gene expression of acetyl-CoA carboxylases in *R. erythropolis* PR4. Moreover, DiO slightly induced the expression of RER_17540 and RER_17550, which encode the beta and the alpha subunits of acetyl-CoA carboxylase, respectively. These differing conclusions from Sabirova's study might be due to the distinct properties of the carbon source used as a control in the two studies (Zheng et al. 2012) or diverse metabolic profiles of the two strains.

In contrast, the type I fatty acid synthase was strongly downregulated in the presence of hydrocarbons; however the suppression of this gene by HeD was much stronger than by DiO. This enzyme is responsible for the synthesis of mid-chain fatty acids ($C_{16–18}$ and $C_{24–26}$) in mycobacteria (Vance et al. 1973; Kikuchi et al. 1992). The downregulation of this megaenzyme can be explained if we assume that substantial amounts of mid-chain fatty acids could be produced from hexadecane, consequently their de novo synthesis could be reduced. This observation coincides with the findings of de Carvalho et al. (2009) who concluded that fatty acids having the same chain length as the alkane substrate can be incorporated into membrane lipids in *R. erythropolis*. In the cells grown on DiO, most of the fatty acids derived from the oxidation of alkanes might not be suitable for incorporation into the membrane without chain length modification. Therefore, the presence of FAS I synthase is necessary for the cells under these conditions.

FAS II is a prokaryotic-type multienzyme system of fatty acid synthesis. In addition to its original function in the majority of prokaryotes, in actinomycetes FAS II adapted to synthesize the long chain meromycolates, precursors of mycolic acid biosynthesis (Choi et al. 2000; Cantaloube et al. 2011). In the final step, meromycolate-ACP synthesized by FAS II is fused to an acyl-CoA in Claisen-type condensation catalyzed by polyketide synthase 13 (Portevin et al. 2004; Gande et al. 2004; Gavalda et al. 2009). The expression of FAS II and the polyketide synthase 13 gene clusters were not affected by hydrocarbons. This might be explained by the crucial role of mycolic acids in the composition of the cell wall in rhodococci and related genera.

We also observed the HeD-related upregulation of the gene clusters coding for enzymes responsible for the biosynthesis of exopolysaccharides and exolipopolysaccharides, coinciding with the findings in *A. borkumensis* (Sabirova et al. 2011). Urai and his co-workers identified and characterized an exopolysaccharide and an exolipopolysaccharide synthesized by *R. erythropolis* PR4 (Urai et al. 2007a, b). These compounds play important roles in biofilm formation, thereby providing protection against the toxicity of organic solvents. They can also be involved in the emulsification of hydrophobic substrates (Iyer et al. 2006; Satpute et al. 2010). However, it was demonstrated here that DiO induced these genes much more than HeD did. This might be due to the toxic characteristics of certain components of diesel oil.

The majority of redox enzymes in *R. erythropolis* PR4 have iron in their active centers; iron chelation and transport via the cell membrane are very important processes during hydrocarbon biodegradation. Several ABC transporter genes were hydrocarbon-inducible. These might be responsible for the

import of iron-bound siderophores. On the other hand, the MFS transporter genes induced by hydrocarbons could produce proteins involved in the export of siderophore molecules. The gene of a non-ribosomal peptide synthase was also induced by hydrocarbons. A recent study showed that this non-ribosomal peptide synthase is responsible for the biosynthesis of heterobactins (Bosello et al. 2013). Heterobactins are iron-siderophore molecules and their production has been reported in *R. erythropolis* and also in *Nocardia tenerifensis* (Carran et al. 2001; Bergeron et al. 2011). The upregulation of iron transporter genes can be interpreted by the increased iron demand of the cell, since the majority of the redox enzymes involved in hydrocarbon biodegradation, contain iron. These results coincide with the findings of Mason and colleagues; they observed expression of iron transport systems in the metatranscriptome of the oil plume caused by the BP Deepwater Horizon oil rig accident (Sabirova et al. 2011; Mason et al. 2012).

The main transcriptomic changes are delineated in Fig. 4. The figure summarizes the most important genes being up- or downregulated by hexadecane (A) and diesel oil (B). The cells grown on HeD maintain several parallel pathways of oxidation of HeD: using (a) AlkB1 and AlkB2; (b) CYP153 (pREL1_0283) and (c) other monooxygenases (MOX). The catabolic routes for DiO are more diverse, including numerous CYP proteins belonging to various families, ring-cleaving dioxygenases and ring-hydroxylases, which are responsible for the conversion of cyclic hydrocarbons. Novel enzymes for alcohol and aldehyde oxidation also appeared. The fatty acids derived from the oxidation of hydrocarbons can be incorporated into the membrane or are subjected to a β-oxidation pathway of elevated activity. The FAS I-related fatty acid biosynthesis was repressed in the presence of HeD, while the genes for several other biosynthetic and transport pathways are induced. These include exopolysaccharide synthesis and transport, as well as iron and K^+ transporters.

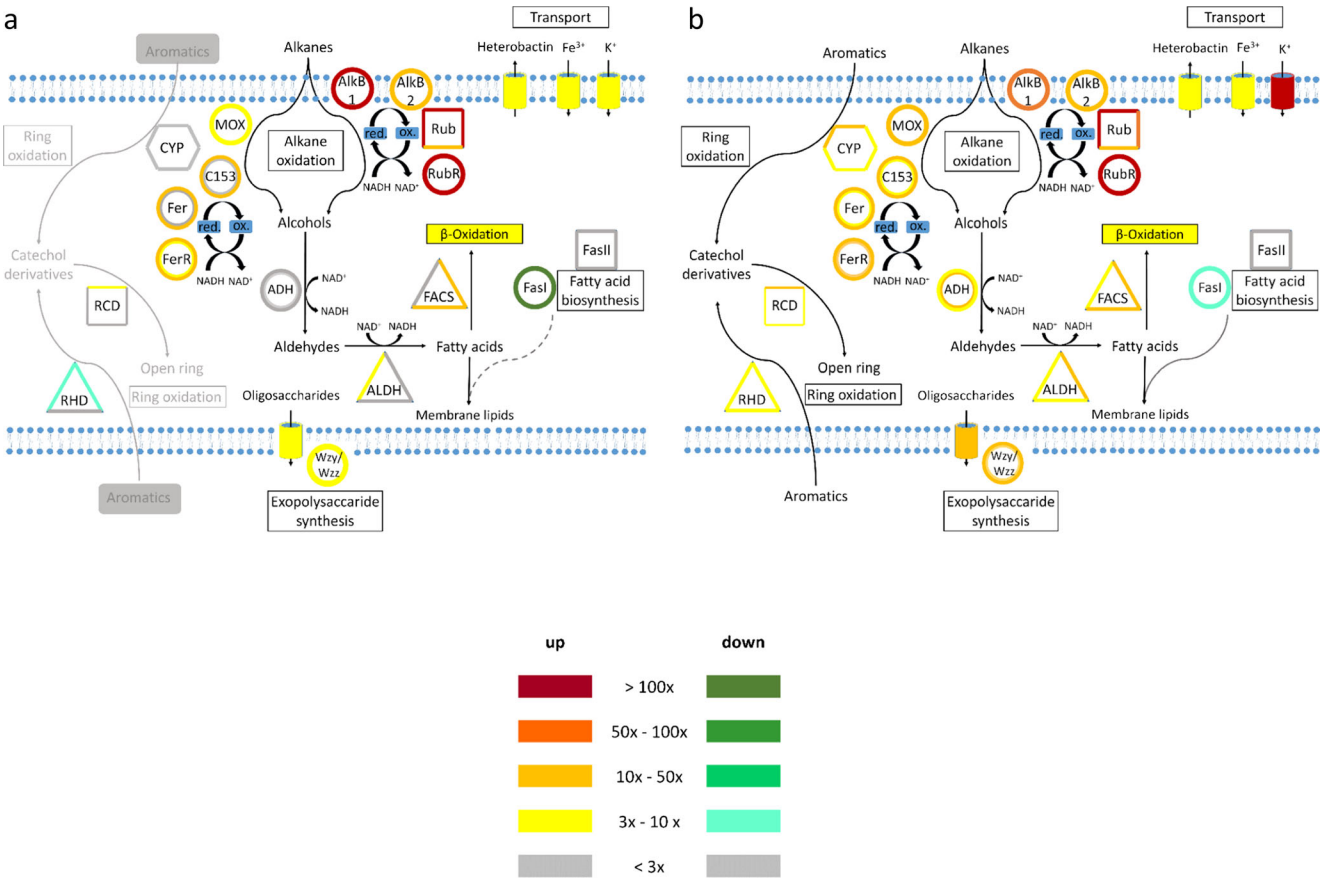

Fig. 4 Illustration of the major metabolic responses of cells grown on acetate, hexadecane and diesel oil. **a** Hexadecane vs acetate, **b** diesel oil vs acetate, Multiple genes of an enzyme type are indicated as follows: *alkB1* and *alkB2* are shown separately. Genes of one or two isoenzymes are shown by (concentric) *circles* with proper color codes. Three isoenzyme genes are shown by a *triangle*, 4–8 genes are displayed as a *tetragon–octagon*, where each edge represent a gene. The *colors of the edges or circles* correspond to the color code shown. *ADH*: alcohol dehydrogenase, *ALDH* aldehyde dehydrogenase, *AlkB1-2* alkane-1-monooxygenase, *Cyp* cytochrome P450 monooxygenase, excepting CYP153 family, *C153* CYP153 family, *FACS* fatty acid-CoA synthase, *FASI-II* fatty acid synthesis proteins, *Fer* ferredoxin, *FerR* ferredoxin reductase, *MOX* other monooxygenases, *RCD* ring-cleaving dioxygenase, *RHD* ring hydroxylating dioxygenase, *Rub* rubredoxin, *RubR* rubredoxin reductase, *Wzy* exopolysaccharide synthesis protein, *Wzz* Wzz domain protein

In addition to the transcription changes displayed in Fig. 4, the expression of numerous hypothetical proteins was significantly changed by the hydrophobic substrates studied. Many of them are located in the vicinity of genes of known function (see Online Table S2). The genes/orfs discussed are highlighted in Online Table S2. Nevertheless, it could be clearly seen that broad and diverse transcriptomic responses could be disclosed in cells exposed to various hydrophobic substrates.

Our transcriptomic analyses identified several novel components, likely involved in diesel degradation and/or having a role in physiological responses. The conclusions expand our view on the metabolic events of oil degradation, provide additional data for functional characterization of rhodococcal genomes and supply important information for up-to-date molecular biomonitoring tools.

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Ethical statement

Compliance with ethical standards

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