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Transcriptomic analysis of the highly efficient oil-degrading bacterium *Acinetobacter venetianus* RAG-1 reveals genes important in dodecane uptake and utilization

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One sentence summary: Analysis of the transcriptome of the oil-degrading bacterium *Acinetobacter venetianus* RAG-1 helps in identification of genes that are involved in uptake and metabolism of alkanes, thus helping in bioremediation.

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ABSTRACT

The hydrocarbonoclastic bacterium *Acinetobacter venetianus* RAG-1 has attracted substantial attention due to its powerful oil-degrading capabilities and its potential to play an important ecological role in the cleanup of alkanes. In this study, we compare the transcriptome of the strain RAG-1 grown in dodecane, the corresponding alkanol (dodecanol), and sodium acetate for the characterization of genes involved in dodecane uptake and utilization. Comparison of the transcriptional responses of RAG-1 grown on dodecane led to the identification of 1074 genes that were differentially expressed relative to sodium acetate. Of these, 622 genes were upregulated when grown in dodecane. The highly upregulated genes were involved in alkane catabolism, along with stress response. Our data suggest AlkMb to be primarily involved in dodecane oxidation. Transcriptional response of RAG-1 grown on dodecane relative to dodecanol also led to the identification of permease, outer membrane protein and thin fimbriae coding genes potentially involved in dodecane uptake. This study provides the first model for key genes involved in alkane uptake and metabolism in *A. venetianus* RAG-1.

Keywords: alkane hydroxylase; alkane monooxygenase; dodecane; alkane uptake; transcriptomic; *Acinetobacter venetianus* RAG-1 ATCC 31012

INTRODUCTION

Release of hydrocarbons into the environment, accidentally or due to industrial practices, is a major cause of environmental pollution. Hence, the hydrocarbonoclastic capacities of various Gammaproteobacteria have drawn attention as a possible strategy for oil spill bioremediation (van Beilen *et al.* 2003, 2007; Wentzel *et al.* 2007). We focus on the degradation of dodecane, which is known to be a contaminant in areas related to fuel spills (Gunasekera *et al.* 2013) and heavy metal mining where it is used as a solvent for radionuclide extraction (Baumgaertner and Finsterwalder 1970; Alibrahim and Shlewit 2007; Nakashima and Kolarik 2007).

The *n*-alkanes are typically functionalized by oxidation of one terminal methyl group to generate the corresponding alcohol by an alkane hydroxylase system. This system consists of three components: an integral membrane protein alkane monooxygenase, AlkB, a soluble NADH-rubredoxin reductase, AlkT, and a soluble rubredoxin, AlkG (Eggink *et al.* 1987; Kok *et al.* 1989; van Beilen *et al.* 2001). Together, these protein components, along with two redox cofactors (NADH and FAD) catalyze the conversion of an alkane to the corresponding alkanol. The alkanol is further oxidized via a pathway involving an alcohol dehydrogenase (AlkJ), aldehyde dehydrogenase (AlkH) and acyl-CoA synthetase (AlkK), followed by the β-oxidation pathway (van Beilen *et al.* 2001).

Aerobic alkane degradation is best characterized in the AlkB-containing *Pseudomonas putida* GPo1 (van Beilen, Wubbolts and Witholt 1994; van Beilen *et al.* 2001). *Acinetobacter* alkane monooxygenases belong to a novel family and are referred to as AlkM instead. Most *Acinetobacters* are known to have two alkane monooxygenases (AlkM) that degrade overlapping ranges of alkanes (generally C9–C40) (van Beilen *et al.* 2003, 2007). The AlkM-based alkane degradation is not well characterized in comparison to AlkB. Given the low amino acid similarity of AlkB and AlkM (13), it is possible that AlkM has a different mechanism of alkane oxidation, which might prove useful in alkane bioremediation under certain conditions.

Previously, Mara *et al.* (2012) found that RAG-1 significantly outperforms 16 other *Acinetobacter* strains in terms of the biomass accumulated when grown on *n*-alkanes. More recently, Fondi *et al.* (2016) have shown that RAG-1 has an exceptional ability to degrade C10–C25 *n*-alkanes. This prompted us to study genes involved in alkane uptake and oxidation in RAG-1. The whole-genome sequence of this strain is available (Fondi *et al.* 2012). It contains two alkane-metabolizing proteins AlkMa and AlkMb with 60% identity to each other. RAG-1 has been widely studied for its ability to produce a potent biosurfactant, emulsan (Rosenberg *et al.* 1982; Pines and Gutnick 1986; Nakar and Gutnick 2001; Peleg *et al.* 2012). Although it has been postulated that emulsan assists in alkane uptake, additional mechanisms that aid uptake and mitigate the potential alcohol toxicity have not been studied.

Microarray-based alkane transcriptional response has been studied in the AlkB-containing strains *Alcanivorax borkumensis* (Sabirova *et al.* 2011) and *P. aeruginosa* strain ATCC 33988 (Gunasekera *et al.* 2013). The alkane transcriptional response of the AlkM-containing *Acinetobacter oleivorans* DR1 identified upregulation of alkane metabolism, fatty acid metabolism, glyoxylate pathway and oxidative stress defense response genes (Jung *et al.* 2015). DR1 harbors two alkane monooxygenases with varied degrees of similarity to the alkane monooxygenases of RAG-1 (Supplementary Information 1, Supporting Information). Unlike RAG-1, the regulator for AlkM could not be identified in

DR1. This suggests differences in their hydrocarbonoclastic phenotype making it imperative to specifically study the powerful alkane-degrading strain RAG-1.

Pairwise comparative analyses were performed on RAG-1 grown in dodecane, dodecanol and sodium acetate (control, hereafter referred to as acetate). Differentially expressed genes important in dodecane degradation were identified. This is the first study to (i) specifically look at transcriptomic response in a hydrocarbonoclastic bacteria grown on dodecane, and (ii) compare gene expression data between cultures grown on an alkane and the corresponding alkanol, obtaining confirmation of the role of *alkMa* and *alkMb* genes in dodecane metabolism along with identification of potential ancillary genes involved in dodecane uptake. Uncovering the genetic determinants responsible for AlkM-based dodecane degradation capacity will be helpful in developing effective bioremediation strategies.

MATERIALS AND METHODS

Additional details can be found in Supplementary Information 2 (Supporting Information).

Bacterial strains and culture conditions

Acinetobacter venetianus RAG-1 (ATCC 31012) was maintained on E2 medium (Brown, Gunasekera and Ruiz 2014) with either 1% v/v dodecane (Smits *et al.* 2002) or 0.01% v/v ethanol (Dams-Kozlowska and Kaplan 2007) at 30°C.

RNA extraction, quantification and library construction

Based on the growth conditions reported in literature (Rosenberg *et al.* 1982; Ratajczak, Geissdörfer and Hillen 1998; Smits *et al.* 2002), RAG-1 was grown in triplicates on three different carbon sources: dodecane (1% v/v), dodecanol (5 mM) and sodium acetate (0.2% w/v). The cells were harvested at mid-log phase. Total RNA was extracted using the Qiagen's RNeasy Mini Kit followed by DNase treatment to eliminate any DNA contamination. RNA obtained was analyzed using the Agilent 2100 Bioanalyzer. The total RNA samples were prepared for Illumina Next-Generation Sequencing using the RiboZero kit and PrepXTM RNA-Seq Library Preparation Kit at the Functional Genomics Lab (QB3-Berkeley Core Research Facility, Berkeley, USA) and sequenced on Illumina HiSeq2000.

RNA-Seq data analysis

The RAG-1 genome (NCBI accession number APPO00000000.1) was uploaded to RAST (Aziz *et al.* 2008; Overbeek *et al.* 2014; Brettin *et al.* 2015) server for annotation. The trimmed, rRNA-depleted RNA-Seq reads were mapped against the RAST-annotated RAG-1 genome using the CLC Bio Genomics Workbench 8.0.2 software (http://www.clcbio.com/products/clcgenomicsworkbench), which re-implemented EdgeR RNA quantification workflow (Robinson, McCarthy and Smyth 2010). Genes exhibiting at least 2-fold change and less than 0.05 false discovery rate (FDR) were considered differentially regulated. The data are accessible through GEO Series accession number GSE78186 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78186) in NCBI's Gene Expression Omnibus (Edgar, Domrachev and Lash 2002). Genes were functionally annotated using the Clusters of Orthologous Groups (COGs) database (Tatusov *et al.* 2000), the SEED subsystems database (Silva *et al.* 2016) and Blast2GO (Conesa *et al.* 2005) to assign Gene Ontology (GO) terms (Ashburner *et al.*

Table 1. The number of differentially expressed genes (exhibiting at least 2-fold change and <0.05 FDR) and the number of upregulated genes amongst them, based on RNA-Seq data in *A. venetianus* RAG-1 grown in dodecane, dodecanol and sodium acetate.

Condition	Genes differentially expressed	Genes upregulated
Dodecane (relative to sodium acetate)	1074	622
Dodecane (relative to dodecanol)	1280	756
Dodecanol (relative to sodium acetate)	785	337

2000). The NCBI reference number and the protein sequence corresponding to the SEED-based Open Reading Frames (ORFs) are listed in Supplementary Information 3 (Supporting Information). Domains were identified using NCBI's conserved domain database (Marchler-Bauer *et al.* 2015).

RESULTS AND DISCUSSION

Functional categories of differentially expressed genes

RNA-Seq was used to compare RAG-1 grown in dodecane, dodecanol or acetate as the sole carbon source. The numbers of genes differentially expressed in the pairwise comparisons are presented in Table 1. The genes upregulated at least 10-fold in the pairwise comparisons could be more significant, and are listed in the Supplementary Information 4 (Supporting Information). Differentially expressed genes were grouped using SEED subsystem-based annotation, the COG gene distribution and GO classification (Supplementary Information 5, Supporting Information).

Core alkane metabolism

Dodecane oxidation

The strain RAG-1 has two alkane monooxygenase-coding genes *alkMa* (ORF_2514) and *alkMb* (ORF_2111) (Fig. 1a). It also has homologs of genes coding for flavin-binding monooxygenase, *almA* (ORF_684) (Wang and Shao 2012). It lacks genes coding for homologs of long-chain alkane monooxygenase *ladA*, and cytochrome P450-related enzymes.

The homolog of *almA* (ORF_684) does not display differential regulation when grown in dodecane relative to acetate or dodecanol. Transcripts of *alkMa* were upregulated 29.8- and 24.2-fold in dodecane relative to acetate and dodecanol, respectively. In comparison, the homolog of *alkMb* was upregulated 150.9- and 41.8-fold in dodecane relative to acetate and dodecanol, respectively. Given the higher fold change, it is possible that AlkMb is primarily involved in dodecane oxidation in RAG-1, with AlkMa providing secondary dodecane-oxidizing capacity. Similarly, in DR1 both *alkM* genes were upregulated in hexadecane with *alkMb* exhibiting higher expression than *alkMa* (Jung *et al.* 2015). The *Acinetobacter* AlkMs might have evolved with overlapping substrate ranges with each performing optimally when degrading a specific range of carbon chain lengths. A 231-bp ORF (ORF_2513) upstream of *alkMb* (Fig. 1a) was also upregulated 157.2- and 35.4-fold when grown in dodecane relative to acetate and dodecanol (Fig. 1b and d), indicating its likely importance in alkane oxidation. The ORF_2513 contains a KTSC domain possibly involved in RNA binding.

Both *alkMa* and *alkMb* genes have proximally encoded regulatory proteins: AlkRa (ORF_2110) and AlkRb (ORF_2515), respectively (Fig. 1a). These regulatory proteins were constitutively expressed in our study, in contrast to *Acinetobacter* sp. strain ADP1 (Ratajczak, Geissdörfer and Hillen 1998). Interestingly, AlkRa and AlkRb are dissimilar proteins based on the domains they encode (41% protein sequence identity; 12% query coverage), suggesting that *alkMa* and *alkMb* are regulated via distinct mechanisms. In *Alcanivorax borkumensis* SK2, the outer membrane protein OmpS detects the presence of alkanes and triggers the expression of an alkane chemotaxis complex (Wang and Shao 2014). No homolog of OmpS was detected in RAG-1.

RAG-1 has homologs of genes encoding rubredoxin (ORF_2811) and rubredoxin reductase (ORF_2812 and ORF_2776). As reported earlier (Ratajczak, Geissdörfer and Hillen 1998; Marin, Yuste and Rojo 2003; Gunasekera *et al.* 2013), these genes do not exhibit differential expression (Fig. 1b and d) when grown on alkanes. As observed in ADP1 (Geißdörfer *et al.* 1999), the genes coding for rubredoxin and rubredoxin reductase, esterase—EstB (ORF_2813) and LysR-type transcriptional regulator related to oxidative stress—OxyR (ORF_2814) constitute an operon (Fig. 1a). None of these genes were differentially expressed in RAG-1 when grown on dodecane relative to acetate.

In *A. borkumensis* SK2 (Sabirova *et al.* 2006), increased expression of cardiolipin synthase (involved in facilitating membrane fusion) was observed in alkane-grown cells. However, the homologs of this enzyme in RAG-1 (ORF_619 and ORF_2277) were not differentially regulated when grown in dodecane relative to acetate.

Dodecanol catabolism

The dodecanol metabolism pathway is expected to be active when RAG-1 is grown in dodecane or dodecanol (Fig. 2). The alcohol dehydrogenase gene, *alkJ*, is involved in alcohol oxidation in GPo1 (Kirmair and Skerra 2014). In RAG-1, its homolog *alkJ* (ORF_2264) displayed 108.3- and 40.0-fold upregulation in dodecane and dodecanol, respectively, relative to acetate supporting a role in dodecanol oxidation. The resulting aldehyde is converted into a fatty acid by aldehyde dehydrogenase, AlkH. The homolog of *alkH* (ORF_2266) showed 35.6- and 10.3-fold upregulation in dodecane and dodecanol, respectively, relative to acetate (Fig. 1b and c). In RAG-1, several ORFs showed varying degrees of similarity to acyl CoA synthetase (AlkK), of which only the ORF_2189 displayed upregulation when grown in both dodecane (3.2-fold) and dodecanol (2.02-fold) relative to acetate, suggesting its involvement in dodecane catabolism. Multiple homologs of the genes involved in the β-oxidation pathway were identified in the genome sequence. Of these, the homologs upregulated in dodecane relative to acetate were acyl-CoA dehydrogenase (ORF_2615, ORF_1860 and ORF_1861), enoyl-CoA hydratase (ORF_1499), 3-hydroxyacyl-CoA dehydrogenase (ORF_116 and ORF_479) and 3-ketoacyl-CoA thiolase (ORF_2186 and ORF_1797).

Dodecane uptake

Alkane uptake mechanisms are not fully understood. They vary based on the species, alkane length and environmental physicochemical features (Wentzel *et al.* 2007; Rojo 2009; Julsing *et al.* 2012; Wang and Shao 2013; Grant *et al.* 2014). We identified the genes potentially involved in dodecane uptake by comparing differentially expressed genes in dodecane relative to dodecanol.

RAG-1 produces emulsan, to emulsify hydrocarbons thereby increasing their bioavailability. It was earlier shown that RAG-1
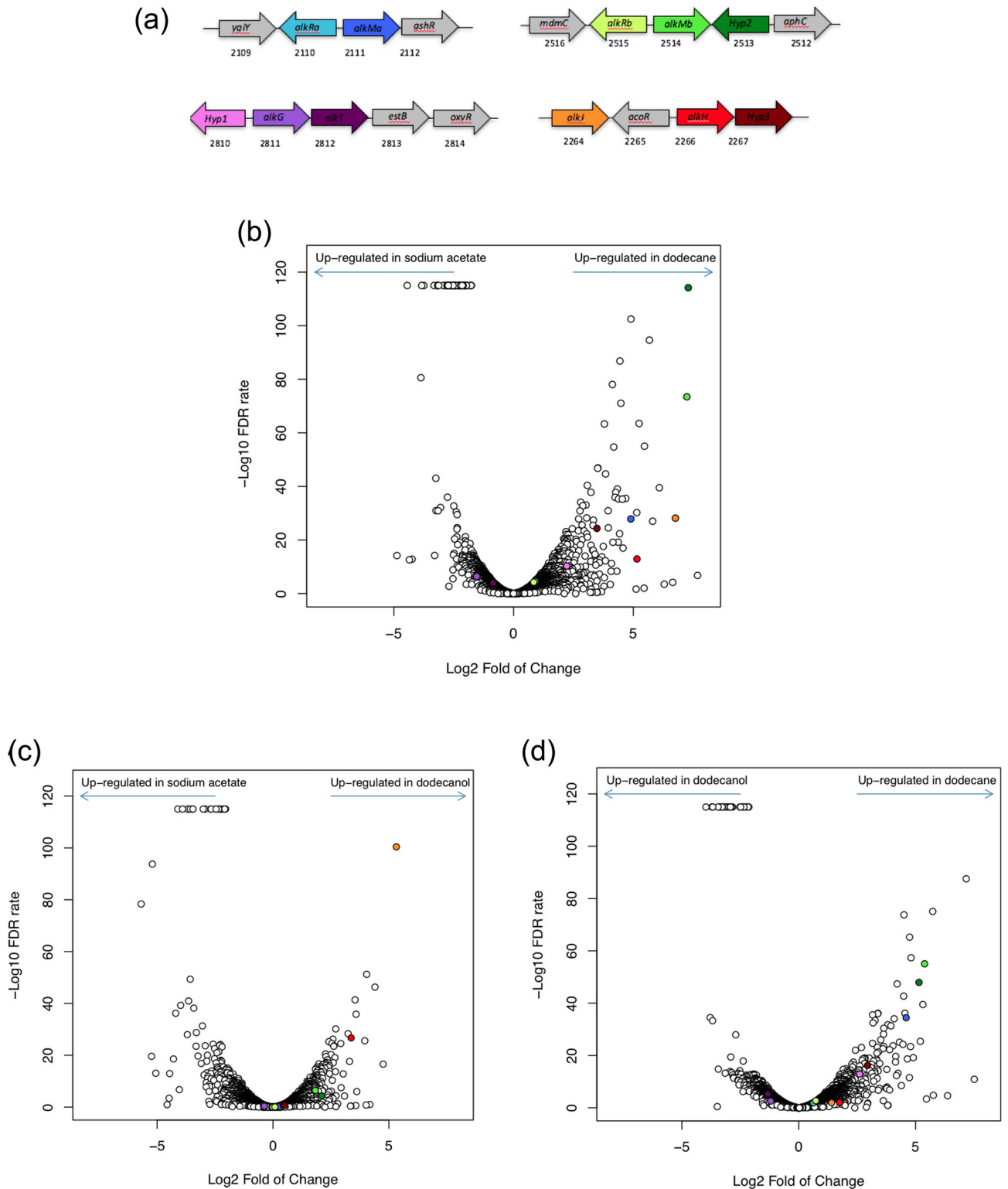
Figure 1. (a) Genetic organization of genes involved in alkane metabolism. Genes depicted—*ygiY*: acyl carrier protein UDP-M acetyl glucosamine O acyl trasferase, *alkRa*: regulator for *alkMa*, *alkMa*: alkane monooxygenase, *gshR*: glutathionine reductase, *Hyp1*: hypothetical protein-coding gene 1, *alkG*: rubredoxin, *alkT*: rubredoxin reductase, *estB*: esterase, *oxyR*: LysR-type transcriptional regulator, *mdmC*: O methyl transferase, *alkRb*: regulator for *alkMb*, *alkMb*: alkane monooxygenase, *Hyp2*: hypothetical protein-coding gene 2, *aphC*: alkyl hydroperoxide reductase protein, *alkJ*: alcohol dehydrogenase, *acoR*: transcriptional activator of acetoin/glycerol metabolism, *alkH*: aldehyde dehydrogenase and *Hyp3*: hypothetical protein-coding gene 3. Volcano plots reporting FDR ($-\log_{10}$FDR) on *y*-axis as a function of log2 fold change on *x*-axis for (**b**) sodium acetate vs. dodecane (**c**) sodium acetate vs. dodecanol and (**d**) dodecane vs. dodecanol. Genes involved in alkane catabolism are highlighted with the color scheme in Fig. 1a.
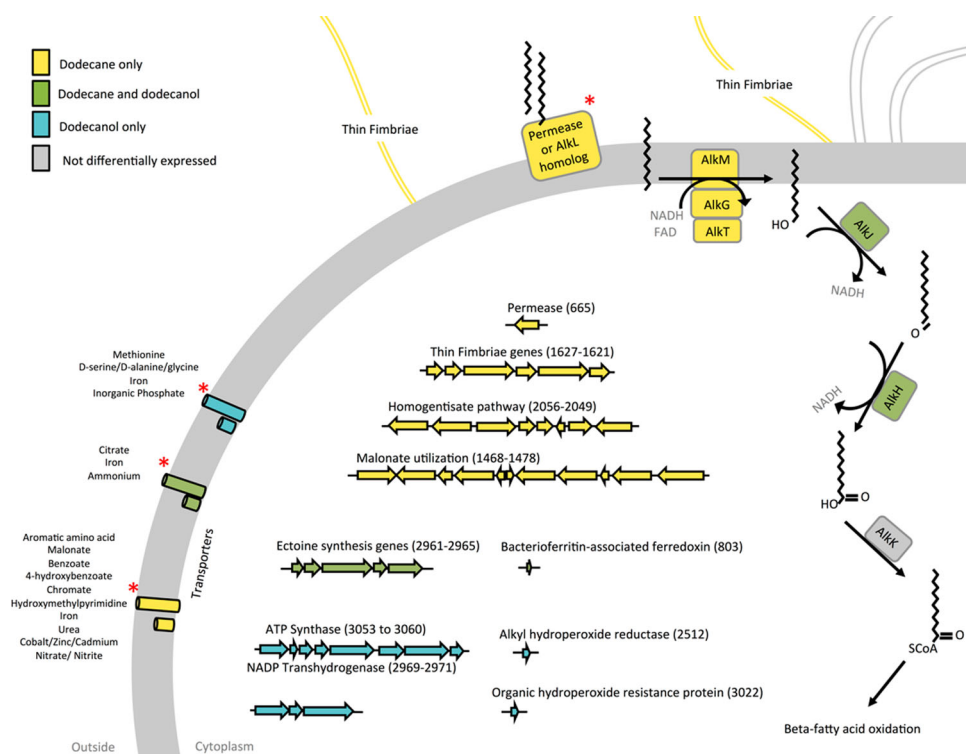
Figure 2. Schematic representation of the transcriptional response to dodecane and dodecanol relative to sodium acetate in *A. venetianus* RAG-1. Yellow represents genes upregulated when grown in dodecane relative to dodecanol and sodium acetate, green represents genes upregulated when grown in dodecane and dodecanol relative to sodium acetate and blue represents genes upregulated when grown in dodecanol relative to dodecane and sodium acetate. The proteins depicted are alkane hydroxylase (AlkM), rubredoxin (AlkG), rubredoxin reductase (AlkT), aldehyde dehydrogenase (AlkH), alcohol dehydrogenase (AlkJ), acyl coenzyme A synthetase (AlkK) and an outer membrane protein putatively involved in alkane transport (AlkL). Sizes are not to scale. Red asterisks refer to the prediction of protein localization not being experimentally confirmed (prediction is based on sequence data).

produces emulsan when grown in hexadecane, ethanol and acetate (Rosenberg *et al.* 1979). Consistent with this report, the emulsan-coding *wee* gene cluster was constitutively expressed, suggesting involvement of other alkane uptake genes, exclusively upregulated in dodecane.

In GPo1, an outer membrane protein, AlkL, is involved in alkane uptake (Julsing *et al.* 2012; Grant *et al.* 2014). The AlkL homolog in DR1 codes for outer membrane protein W, OmpW (26% amino acid identity to AlkL). It displays upregulation in hexadecane (Jung *et al.* 2015) and was hypothesized to aid in alkane uptake in DR1. The gene coding for OmpW (ORF_1329) in RAG-1 displayed 22% amino acid identity to AlkL in Gpo1 and was upregulated (3.5-fold) when grown in dodecane relative to acetate. Interestingly, this gene was also upregulated (4.2-fold) when grown in dodecane relative to dodecanol, strengthening its role in dodecane uptake.

Unlike previous reports in *Pseudomonas aeruginosa* (Gunasekera *et al.* 2013), an upregulation of genes involved in biofilm formation when grown in alkanes was not observed in RAG-1. In *A. borkumensis* SK2, the lipoprotein-releasing proteins (Lol proteins) involved in targeting and anchoring lipoproteins, along with biosurfactant release, are upregulated when grown on alkanes (Sabirova *et al.* 2006). Its homologs (ORF_339, ORF_1557, ORF_1251, ORF_1252) in RAG-1 were not differentially expressed when grown on dodecane (relative to acetate or dodecanol). In *A. borkumensis* SK2, an outer membrane lipoprotein, is proposed to be directly involved in alkane uptake (Sabirova *et al.* 2011). However, its homolog in RAG-1 (ORF_2233) did not show differential expression.

In RAG-1, an 849-bp ORF with homology to a permease (ORF_665) was highly expressed (17.7-fold) when grown in dodecane relative to acetate. This transcript was also upregulated when grown in dodecane relative to dodecanol (17.6-fold), making it a candidate protein potentially involved in mediation of dodecane transport. The ORF_665 has no close homologs in other hydrocarbonoclastic bacteria, so if it does function in alkane uptake, it could be a trait unique to the RAG-1 strain. A 5019-bp hypothetical protein (ORF_664) located upstream of this permease was upregulated 22-fold when grown in dodecane relative to dodecanol (26-fold in dodecane relative to acetate). ORF_664 has no putative annotated domains, but exhibits identity to certain *Acinetobacter* membrane proteins. These genes are interesting candidates for further physiological and functional investigation. Membrane proteins and permeases upregulated in dodecane relative to dodecanol might be important in dodecane uptake, or the uptake of nutrients/cofactors required for dodecane oxidation (Table 2).

Thin fimbriae are postulated to enable RAG-1 to adhere to hydrophobic surfaces like *n*-alkane droplets, rendering these accessible for cellular uptake. There are multiple gene clusters coding for fimbriae/pilus in RAG-1. To the best of our knowledge, the genes coding for thin fimbriae involved in alkane uptake, have not been identified. We found a pilus-coding gene cluster (ORF_1622-ORF_1627) exclusively upregulated (2.4–8.9-fold) in dodecane relative to dodecanol and acetate. These genes are also clustered in the alkane-degrading strain *Acinetobacter baumanni* AB307-0294. It is possible that this gene cluster codes for the thin fimbriae that aid in the alkane uptake in RAG-1.

Table 2. Permeases and membrane proteins upregulated (fold change > 2, FDR < 0.05) in dodecane relative to dodecanol in *A. venetianus* RAG-1.

ORF	Gene product	Fold change
665	Permease	17.6
634	Permease of the drug/metabolite transporter DMT superfamily	5.7
174	Permease of the drug/metabolite transporter DMT superfamily	3.3
986	Urea ABC transporter, permease protein, UrtB	3.1
2474	Urea carboxylase-related ABC transporter, permease protein	3.0
2966	MFS permease protein	2.8
676	TRAP-type C4-dicarboxylate transport system, large permease component	2.8
2082	Permease of the drug/metabolite transporter DMT superfamily	2.8
2562	Histidine transport protein permease	2.7
2234	Arginine permease, RocE	2.3
3045	Permease of the drug/metabolite transporter DMT superfamily	2.1
2581	Xanthine permease	2.0
1111	Integral membrane protein	16.9
1329	Outer membrane protein W	4.2
224	Probable membrane protein	3.7
2767	Probable glutathione S-transferase-related transmembrane protein	3.6
2945	Outer membrane receptor proteins, mostly Fe transport	3.4
894	Integral membrane protein	3.3
1465	RND efflux system, outer membrane lipoprotein, CmeC	3.3
2247	Membrane fusion component of tripartite multidrug resistance system	3.2
2886	Outer membrane protein A precursor	3.1
948	Probable transmembrane protein	3.0
1652	Probable transmembrane protein	3.0
2644	Heavy metal RND efflux outer membrane protein, CzcC family	2.9
2847	Putative outer membrane protein	2.8
2325	Putative iron-regulated membrane protein	2.7
1457	Predicted membrane fusion protein MFP component of efflux pump, membrane anchor protein, YbhG	2.7

Table 3. Transporters upregulated in dodecane (relative to dodecanol and sodium acetate), dodecane and dodecanol (relative to sodium acetate), and dodecanol (relative to dodecane and sodium acetate) in *A. venetianus* RAG-1.

Condition	Transport proteins upregulated
Dodecane	Permease of the drug/metabolite transporter DMT superfamily (ORF_634), malonate transporter, MadL (ORF_1470) and MadM (ORF_1469), benzoate MFS transporter BenK (ORF_2262), benzoate transport protein (ORF_2257), ABC transporter ATP-binding protein (ORF_2300), aromatic amino acid transport protein (ORF_2055), 4-hydroxybenzoate transporter (ORF_2591), urea ABC transporter, urea-binding protein (ORF_987), urea carboxylase-related ABC transporter, permease protein (ORF_2474), hydroxymethylpyrimidine ABC transporter, substrate-binding component (ORF_2573), nitrate/nitrite transporter (ORF_1328), chromate transport protein ChrA (ORF_1204), cobalt/zinc/cadmium efflux RND transporter membrane fusion protein, CzcB family (ORF_2643), zinc ABC transporter periplasmic-binding protein, ZnuA (ORF_3062)
Dodecane and dodecanol	Periplasmic phosphate-binding protein PstS (ORF_1434), iron compound ABC uptake transporter permease protein (ORF_2445), citrate transporter (ORF_394), ammonium transporter (ORF_360)
Dodecanol	Methionine transporter (ORF_497), RND efflux system, inner membrane transporter CmeB (ORF_347), low-affinity inorganic phosphate transporter (ORF_1223), D-serine/D-alanine/glycine transporter (ORF_1228), ferrous transport protein (ORF_64), iron compound ABC uptake transporter ATP-binding protein (ORF_2443), iron compound ABC uptake transporter substrate-binding protein (ORF_2442)

Other significantly responsive genes

As reported in previous gene expression studies (Gunasekera *et al.* 2013; Jung *et al.* 2015), an upregulation of genes homologous to iron uptake genes (ORF_2445, ORF_2118 and ORF_2945) was seen when RAG-1 was grown in dodecane relative to acetate. This is expected since the alkane monooxygenase is known to possess an iron-containing core.

Genes upregulated when grown on both dodecane and dodecanol relative to acetate are likely important in dodecanol metabolism. These genes encoded ectoine biosynthesis, bacterioferritin-associated ferredoxin and certain transporters (Table 3). Genes upregulated in dodecane relative to dodecanol might be important in alkane uptake and oxidation (Table 4).

Conversely, genes upregulated when grown in dodecanol relative to dodecane are most likely involved in alcohol uptake

Table 4. Other genes of interest (not including genes coding for core alkane metabolism) highly upregulated in dodecane relative to dodecanol, dodecane and dodecanol relative to sodium acetate, and dodecanol relative to dodecane in *A. venetianus* RAG-1.

Upregulated in dodecane relative to dodecanol

Functional annotation	ORFs upregulated	Annotated function/possible role
Hypothetical protein	2947 (182.6-fold)	Unknown
Hypothetical protein	594 (54.3-fold)	Unknown
Hypothetical proteins	1533 (143.5-fold) 1534 (28.0-fold)	Metal-dependent hydrolase. Closest homologs in *A. baumannii*, *P. aeruginosa* PAO1 and *A. borkumensis* SK2, indicating their importance in alkane-metabolizing strains.
ThiJ/PfpI family protein	2229 (82.5-fold)	Putative function of intracellular protease/amidase based on ThiJ domain. Chaperone and stress response proteins based on GATase1-like domain
Homogentisate pathway	2049 (14.0-fold) 2053 (23.3-fold) 2054 (53.2-fold) 2055 (5.4-fold) 2056 (2.2-fold)	Aromatic compound degradation, including aromatic amino acids such as tyrosine and phenylalanine, found in peptide-utilizing hyperthermophilic *Archaea* (Mai and Adams 1994; Siddiqui, Fujiwara and Imanaka 1997; Mardanov *et al.* 2009). Upregulation also observed in other alkane-degrading strains (Palleroni, Pieper and Moore 2010; Lincoln *et al.* 2015).
Malonate utilization	1468–1478 (2.1–22.7- fold)	Malonate transport into the cell, and decarboxylation to acetate and carbon dioxide

Upregulated in both dodecane and dodecanol relative to sodium acetate

Functional annotation	ORFs upregulated	Annotated function/possible role
Ectoine synthesis	2961–2965 (14.3–29.9-fold in dodecane; 5.3–11.6-fold in dodecanol)	Compatible solute (Kuhlmann and Bremer 2002), possibly provides protection against oxidative stress (Andersson, Breccia and Hatti-Kaul 2000).
Bacterioferritin-associated ferredoxin, *bfd*	803 (21.5-fold in dodecane and 26.4-fold in dodecanol)	This gene is most often proximal to bacterioferritin *bfr*. The genes bfd and bfr are reciprocally regulated, such that iron starvation induces *bfd* expression but represses *bfr* expression (Quail *et al.* 1996). Bfd is hypothesized to be involved in the insertion of iron into heme (Quail *et al.* 1996) and may be important for cells expressing the iron-containing alkane monooxygenases.

Upregulated in dodecanol relative to dodecane

Functional annotation	ORFs upregulated	Possible role
Alkyl hydroperoxide reductase, AhpC	2512 (13.2-fold)	Oxidative stress response protein
Organic hydroperoxide resistance protein	3022 (12.9-fold)	Oxidative stress response protein
NADP transhydrogenase	2969–2971 (8.0– 9.7-fold)	Catalyzes the conversion between NADPH and NADH. The enzyme is also known to protect cells from oxidative stress (Kowaltowski, Castilho and Vercesi 2001).
ATP synthase	3053–3060 (4.6–7.7-fold)	ATP synthesis

or alcohol stress response. The SEED annotation confirms that genes involved in stress response were highly upregulated when grown in dodecanol (Table 4). Other genes significantly upregulated in dodecanol include the ORF_1050-ORF_1052, which are also clustered in other alkane-metabolizing bacteria such as *P. aeruginosa* PAO1, *P. fluorescens* SBW25 and *A. borkumensis* SK2. In addition, genes coding for the putative membrane protein (ORF_1238) and putative permease (ORF_ 2440) are significantly upregulated in externally supplied dodecanol, suggesting their possible role in alkanol uptake.

Acetate metabolism

Growth on acetate was marked by 5.8-fold upregulation of acetate permease, *actP* (ORF_3034) relative to dodecane. The acetate permease is expected to be involved in the uptake of acetate. This acetate is likely phosphorylated to acetyl-CoA, by acetate kinase and phosphate acetyltransferase. The homologs of acetate kinase (ORF_632) and phosphate acetyltransferase (ORF_633) were upregulated 4.1- and 4.6-fold when grown in acetate relative to dodecane. It is known that the glyoxylate bypass pathway is essential for growth on carbon substrates such as acetate since it allows conversion of acetyl-CoA to metabolic intermediates (Kornberg 1966). Accordingly, the ORFs coding for the enzymes citrate synthase (ORF_313), aconitase (ORF_2449), isocitrate lyase (ORF_2800), malate synthase (ORF_2347) and malate dehydrogenase (ORF_290) were upregulated 6.6-, 10.0-, 21.8-, 2.7- and 6.9-fold in acetate relative to dodecane.

CONCLUSIONS

We report the first comprehensive transcriptome analysis of the highly efficient alkane-degrading strain RAG-1. This strain encodes three genes involved in alkane oxidation: *alkMa*, *alkMb* and *almA*. The gene *alkMb* demonstrated the highest differential expression and may be primarily involved in dodecane oxidation. It is likely that AlkMa also possesses the capability to oxidize dodecane. Given that *almA* was not differentially expressed, it might not be involved in dodecane oxidation. Since the hypothetical protein located next to *alkMb* is very highly upregulated, future studies should include this gene when attempting heterologous expression of alkane monooxygenase from RAG-1. The genes

coding for a permease, an outer membrane protein and thin fimbriae were implicated in dodecane uptake. This study provides a functional understanding of pathways involved in dodecane uptake and metabolism in the strain RAG-1 beyond annotations, and the data is useful for utilizing this metabolism natively or reconstituting it in another host.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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REFERENCES

Alibrahim M, Shlewit H. Solvent extraction of uranium (VI) by tributyl phosphate/dodecane from nitric acid medium. *Period Polytech Chem* 2007;**51**:57–60.

Andersson MM, Breccia JD, Hatti-Kaul R. Stabilizing effect of chemical additives against oxidation of lactate dehydrogenase. *Biotechnol Appl Biochem* 2000;**32**:145–153.

Ashburner M, Ball CA, Blake JA *et al.* Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000;**25**:25–9.

Aziz RK, Bartels D, Best AA *et al.* The RAST Server: Rapid Annotations using Subsystems Technology. *BMC Genomics* 2008;**9**:75.

Baumgaertner F, Finsterwalder L. On the transfer mechanism of uranium(VI) and plutonium(IV) nitrate in the system nitric acid-water/tributylphosphate-dodecane. *J Phys Chem* 1970;**74**:108–12.

Brettin T, Davis JJ, Disz T *et al.* RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Sci Rep* 2015;**5**:8365.

Brown LM, Gunasekera TS, Ruiz ON. Draft genome sequence of *Pseudomonas aeruginosa* ATCC 33988, a bacterium highly adapted to fuel-polluted environments. *Genome Announc* 2014;**2**, DOI: 10.1128/genomeA.01113-14.

Conesa A, Götz S, García-Gómez JM *et al.* Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 2005;**21**:3674–6.

Dams-Kozlowska H, Kaplan DL. Protein engineering of Wzc to generate new emulsan analogs. *Appl Environ Microb* 2007;**73**:4020–8.

Edgar R, Domrachev M, Lash A. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 2002;**30**:207–10.

Eggink G, van Lelyveld PH, Arnberg A *et al.* Structure of the *Pseudomonas putida* alkBAC operon. Identification of transcription and translation products. *J Biol Chem* 1987;**262**:6400–6.

Fondi M, Maida I, Perrin E *et al.* Genomic and phenotypic characterization of the species *Acinetobacter venetianus*. *SciRep* 2016;**6**.

Fondi M, Orlandini V, Emiliani G *et al.* Draft genome sequence of the hydrocarbon-degrading and emulsan-producing strain *Acinetobacter venetianus* RAG-1T. *J Bacteriol* 2012;**194**:4771–2.

Geißdörfer W, Kok RG, Ratajczak A *et al.* The genes *rubA* and *rubB* for alkane degradation in *Acinetobacter* sp. strain ADP1 are in an operon with *estB*, encoding an esterase, and *oxyR*. *J Bacteriol* 1999;**181**:4292–8.

Grant C, Deszcz D, Wei Y-C *et al.* Identification and use of an alkane transporter plug-in for applications in biocatalysis and whole-cell biosensing of alkanes. *Sci Rep* 2014;**4**:5844.

Gunasekera TS, Striebich RC, Mueller SS *et al.* Transcriptional profiling suggests that multiple metabolic adaptations are required for effective proliferation of *Pseudomonas aeruginosa* in jet fuel. *Environ Sci Technol* 2013;**47**:13449–58.

Julsing MK, Schrewe M, Cornelissen S *et al.* Outer membrane protein AlkL boosts biocatalytic oxyfunctionalization of hydrophobic substrates in *Escherichia coli*. *Appl Environ Microb* 2012;**78**:5724–33.

Jung J, Jang, IA, Ahn S *et al.* Molecular mechanisms of enhanced bacterial growth on hexadecane with red clay. *Microb Ecol* 2015;**70**:912–21.

Kirmair L, Skerra A. Biochemical analysis of recombinant AlkJ from *Pseudomonas putida* reveals a membrane-associated, flavin adenine dinucleotide-dependent dehydrogenase suitable for the biosynthetic production of aliphatic aldehydes. *Appl Environ Microb* 2014;**80**:2468–77.

Kok M, Oldenhuis R, van der Linden MP *et al.* The *Pseudomonas oleovorans* alkBAC operon encodes two structurally related rubredoxins and an aldehyde dehydrogenase. *J Biol Chem* 1989;**264**:5442–51.

Kornberg HL. The role and control of the glyoxylate cycle in *Escherichia coli*. *Biochem J* 1966;**99**:1–11.

Kowaltowski AJ, Castilho RF, Vercesi AE. Mitochondrial permeability transition and oxidative stress. *FEBS Lett* 2001;**495**:12–15.

Kuhlmann AU, Bremer E. Osmotically regulated synthesis of the compatible solute ectoine in Bacillus pasteurii and related Bacillus spp. *Appl Environ Microbiol* 2002;**68**:772–83.

Lincoln SA, Hamilton TL, Valladares Juárez AG *et al.* Draft genome sequence of the piezotolerant and crude oil-degrading bacterium *Rhodococcus qingshengii* strain TUHH-12. *Genome Announc* 2015;**3**:e00268–15.

Mai X, Adams MW. Indolepyruvate ferredoxin oxidoreductase from the hyperthermophilic archaeon *Pyrococcus furiosus*. A new enzyme involved in peptide fermentation. *J Biol Chem* 1994;**269**:16726–32.

Mara K, Decorosi F, Viti C *et al.* Molecular and phenotypic characterization of *Acinetobacter* strains able to degrade diesel fuel. *Res Microbiol* 2012;**163**:161–72.

Marchler-Bauer A, Derbyshire MK, Gonzales NR *et al.* CDD: NCBI's conserved domain database. *Nucleic Acids Res* 2015;**43**:D222–6.

Mardanov AV, Ravin NV, Svetlitchnyi NV *et al.* Metabolic versatility and indigenous origin of the archaeon *Thermococcus sibiricus*, isolated from a siberian oil reservoir, as revealed by genome analysis. *Appl Environ Microbiol* 2009;**75**:4580–8.

Marin MM, Yuste L, Rojo F. Differential Expression of the Components of the Two Alkane Hydroxylases from *Pseudomonas aeruginosa*. *J Bacteriol* 2003;**185**:3232–7.

Nakar D, Gutnick DL. Analysis of the *wee* gene cluster responsible for the biosynthesis of the polymeric bioemulsifier from the oil-degrading strain *Acinetobacter lwoffii* RAG-1. *Microbiology* 2001;**147**:1937–46.

Nakashima T, Kolarik Z. The formation of a third phase in the simultaneous extraction of actinide (IV) and uranyl nitrates by tributyl phosphate in dodecane. *Solvent Extr Ion Exch* 2007;**1**:497–513.

Overbeek R, Olson R, Pusch GD *et al*. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res* 2014;**42**:D206–14.

Palleroni NJ, Pieper DH, Moore ERB. *Handbook of Hydrocarbon and Lipid Microbiology*. Timmis KN (ed.). Berlin, Heidelberg: Springer, 2010.

Peleg AY, de Breij A, Adams MD *et al*. The success of *Acinetobacter* species; genetic, metabolic and virulence attributes. *PLoS One* 2012;**7**:e46984.

Pines O, Gutnick D. Role for emulsan in growth of *Acinetobacter calcoaceticus* RAG-1 on crude oil. *Appl Environ Microb* 1986;**51**:661–3.

Quail MA, Jordan P, Grogan JM *et al*. Spectroscopic and voltammetric characterisation of the bacterioferritin-associated ferredoxin of *Escherichia coli*. *Biochem Biophys Res Commun* 1996;**229**:635–42.

Ratajczak A, Geissdörfer W, Hillen W. Expression of alkane hydroxylase from *Acinetobacter* sp. Strain ADP1 is induced by a broad range of *n*-alkanes and requires the transcriptional activator AlkR. *J Bacteriol* 1998;**180**:5822–7.

Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 2010;**26**:139–40.

Rojo F. Degradation of alkanes by bacteria. *Environ Microbiol* 2009;**11**:2477–90.

Rosenberg E, Zuckerberg A, Rubinovitz C *et al*. Emulsifier of Arthrobacter RAG-1: isolation and emulsifying properties. *Appl Environ Microb* 1979;**37**:402–8.

Rosenberg M, Bayer EA, Delarea J *et al*. Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. *Appl Environ Microb* 1982;**44**:929–37.

Sabirova JS, Becker A, Lünsdorf H *et al*. Transcriptional profiling of the marine oil-degrading bacterium *Alcanivorax borkumensis* during growth on *n*-alkanes. *FEMS Microbiol Lett* 2011;**319**:160–8.

Sabirova JS, Ferrer M, Regenhardt D *et al*. Proteomic insights into metabolic adaptations in *Alcanivorax borkumensis* induced by alkane utilization. *J Bacteriol* 2006;**188**:3763–73.

Siddiqui MA, Fujiwara S, Imanaka T. Indolepyruvate ferredoxin oxidoreductase from Pyrococcus sp. KOD1 possesses a mosaic structure showing features of various oxidoreductases. *Mol Gen Genet* 1997;**254**:433–439.

Silva GGZ, Green KT, Dutilh BE *et al*. SUPER-FOCUS: A tool for agile functional analysis of shotgun metagenomic data. *Bioinformatics* 2016;**32**:354–61.

Smits THM, Balada SB, Witholt B *et al*. Functional Analysis of Alkane Hydroxylases from Gram-Negative and Gram-Positive Bacteria. *J Bacteriol* 2002;**184**:1733–42.

Tatusov RL, Galperin MY, Natale DA *et al*. The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res* 2000;**28**:33–6.

van Beilen JB, Funhoff EEG, van Beilen J *et al*. Alkane hydroxylases involved in microbial alkane degradation. *Appl Microbiol Biot* 2007;**74**:13–21.

van Beilen JB, Li Z, Duetz Wa *et al*. Diversity of Alkane hydroxylase systems in the environment. *Oil Gas Sci Technol* 2003;**58**:427–40.

van Beilen JB, Panke S, Lucchini S *et al*. Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the alk genes. *Microbiology* 2001;**147**:1621–30.

van Beilen JB, Wubbolts M, Witholt B. Genetics of alkane oxidation by *Pseudomonas oleovorans*. Biodegradation 1994;**5**:161–74.

Wang W, Shao Z. Diversity of flavin-binding monooxygenase genes (*almA*) in marine bacteria capable of degradation long-chain alkanes. *FEMS Microbiol Ecol* 2012;**80**:523–33.

Wang W, Shao Z. Enzymes and genes involved in aerobic alkane degradation. *Front Microbiol* 2013;**4**, DOI: 10.3389/fmicb.2013.00116.

Wang W, Shao Z. The long-chain alkane metabolism network of *Alcanivorax dieselolei*. *Nat Commun* 2014;**5**:5755.

Wentzel A, Ellingsen TTE, Kotlar HH-K *et al*. Bacterial metabolism of long-chain *n*-alkanes. *Appl Microbiol Biot* 2007;**76**:1209–21.