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Permalink
https://escholarship.org/uc/item/1nh7p66q

Journal
FEMS microbiology letters, 363(20)

ISSN
0378-1097

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Publication Date
2016-10-01

DOI
10.1093/femsle/fnw224

Peer reviewed
RESEARCH LETTER – Physiology & Biochemistry

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.

Editor: Hermann Heipieper

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 Finsen 1970; Alibrahim and Shlewi 2007; Nakashima and Karlik 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 monoxygenase, 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), an aldehyde dehydrogenase (AlkH) and an 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 Gp01 (van Beilen, Wubbelts and Witholt 1994; van Beilen et al. 2001). Acinetobacter alkane monoxygenases belong to a novel family and are referred to as AlkM instead. Most Acinetobacters are known to have two alkane monoxygenases (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 AlkB 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 AlkMc-containing Acinetobacter oleivorans DR1 identified upregulation of alkane metabolism, fatty acid metabolism, glycolysis pathway and oxidative stress defense response genes (Jung et al. 2015). DR1 harbors two alkane monoxygenases with varied degrees of similarity to the alkane monoxygenases of RAG-1 (Supplementary Information 1, Supporting Information). Unlike RAG-1, the regulator for AlkMc 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. 2000).
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 monoxygenase-coding genes alkMa (ORF_2514) and alkMb (ORF_2111) (Fig. 1a). It also has homologs of genes coding for flavin-binding monoxygenase, almA (ORF_684) (Wang and Shao 2012). It lacks genes coding for homologs of long-chain alkane monoxygenase 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 AlkBMB 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 AlkBMs 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 (Fig. 1a and d). None of these genes were differentially expressed when RAG-1 is grown in dodecane relative to acetate.

Multiple homologs of this enzyme in RAG-1 (ORF_619 and ORF_2277) were not differentially regulated when grown in dodecane relative to acetate.

Kothari et al. | 3

Dodecanol 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; Juising et al. 2012; Wang and Shao 2013; Grant et al. 2014). We identified the genes potentially involved in dodecanol uptake by comparing differentially expressed genes in dodecanol relative to dodecanol.

RAG-1 produces emulsion, 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 transferase, alkRa: regulator for alkMa, alkMa: alkane monooxygenase, gshR: glutathione 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 monooxygenase (AlkM), rubredoxin (AlkG), rubredoxin reductase (AlkT), aldehyde dehydrogenase (AlkH), alcohol dehydrogenase (AlkJ), acylcoenzyme 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 (17.5-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 baumannii 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 monoxygenase 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 dodecanol, dodecane and dodecanol relative to sodium acetate, and dodecanol relative to dodecane in A. venetianus RAG-1.

### Upregulated in dodecanol relative to dodecane

| 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. |
| Thiol/Pfpl family protein | 2229 (82.5-fold) | Putative function of intracellular protease/amidase based on Thiol 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 dodecanol and dodecane relative to sodium acetate

| Functional annotation | ORFs upregulated | Annotated function/possible role |
|-----------------------|-----------------|----------------------------------|
| Ectoine synthesis | 2961–2965 (14.3–29.9-fold in dodecanol; 5.3–11.6-fold in dodecane) | 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 dodecanol and 26.4-fold in dodecano) | 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.

FUNDING

This work was supported by funding from the Lawrence Livermore National Lab (LLNL) via US Department of Energy, Office of Science, Office of Biological and Environmental Research Contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the US Department of Energy [LBNL Award No.: IC009952, Sponsor Award No.: B604346, WFO B&R Code: YN1901000]. This work was performed under the auspices of the US Department of Energy by LLNL under Contract DE-AC52-07NA27344. The US Government retains and the publisher, by accepting the article for publication, acknowledges that the US Government retains a non-exclusive, paid-up, irrevocable, worldwide license to publish or reproduce the published form of this manuscript, or allow others to do so, for US Government purposes.

Conflict of interest. None declared.

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