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Proteomic insights into metabolic adaptations in *Alcanivorax*  
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1 **Proteomic insights into metabolic adaptations in *Alcanivorax borkumensis***  
2 **induced by alkane utilization**

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## 1 **Abstract**

2           *Alcanivorax borkumensis* (*Abo*) is a ubiquitous marine petroleum oil-degrading  
3 bacterium with an unusual physiology specialized for alkane metabolism. This  
4 “hydrocarbonoclastic” bacterium degrades an exceptionally broad range of alkane  
5 hydrocarbons, but few other substrates. The proteomic analysis presented here reveals  
6 metabolic features of the “hydrocarbonoclastic” lifestyle. Specifically, hexadecane-grown  
7 and pyruvate-grown cells differed in expression of 97 cytoplasmic and membrane-associated  
8 proteins whose genes appeared to be components of 46 putative operon structures.  
9 Membrane proteins up-regulated in alkane-grown cells included three enzyme systems able  
10 to convert alkanes via terminal oxidation to fatty acids, namely enzymes encoded by the  
11 well-known *alkB1* gene cluster, and two new alkane hydroxylating systems, a *P<sub>450</sub>*  
12 cytochrome monooxygenase and a putative flavin-binding monooxygenase, and enzymes  
13 mediating  $\beta$ -oxidation of fatty acids. Cytoplasmic proteins up-regulated in hexadecane-  
14 grown cells reflect a central metabolism based on a fatty acid diet: namely enzymes of the  
15 glyoxylate bypass and of the gluconeogenesis pathway, able to provide key metabolic  
16 intermediates, like phosphoenolpyruvate, from fatty acids. They also include enzymes for  
17 synthesis of riboflavin and of unsaturated fatty acids and cardiolipin, which presumably  
18 reflect membrane restructuring required for membranes to adapt to perturbations induced by  
19 the massive influx of alkane oxidation enzymes. Ancillary functions up-regulated included  
20 the lipoprotein releasing system (Lol), presumably associated with biosurfactant release, and  
21 polyhydroxyalkanoate synthesis enzymes associated with carbon storage under conditions of  
22 carbon surfeit. The existence of three different alkane-oxidizing systems is consistent with  
23 the broad range of oil hydrocarbons degraded by *Abo* and its ecological success in oil-  
24 contaminated marine habitats.

## 1 **Introduction**

2 *Alcanivorax borkumensis* (*Abo*) is a key marine oil-degrading bacterium that can  
3 dramatically increase in numbers after an oil spill and become the most abundant microbe in  
4 oil-polluted waters (23, 28, 29, 45). The list of sites where it has been isolated and shown to  
5 be involved in oil degradation grows with microbiological investigations of oil spills (39, 11,  
6 Yakimov, personal communication). The physiology of *A. borkumensis* is characterized by  
7 oligotrophy and a highly restricted growth substrate profile, namely petroleum hydrocarbons  
8 plus a few organic acids, though the spectrum of hydrocarbons degraded is exceptionally  
9 broad. Its unusual metabolic features, presumed global importance in the natural biological  
10 removal of oil entering marine systems, and biotechnological potential for mitigation of the  
11 ecological damage caused by oil spills, stimulated recent and current functional genomic  
12 studies of this organism. A key question concerning the ubiquity and competitive success of  
13 *Abo* in many marine locations is the genomic and biochemical basis of its physiological  
14 specialization and broad hydrocarbon substrate spectrum.

15 Hydrocarbon degradation is generally initiated by monooxygenases, encoded by *alkB*  
16 genes, which are widely present in oil degrading Gram-negative bacteria (43). Additional  
17 alkane hydroxylating systems, such as alkane hydroxylase systems belonging to the  
18 cytochrome P<sub>450</sub> family, have been found in *Rhodococcus rhodochrous* ATCC 19067 (6)  
19 and *Acinetobacter calcoaceticus* EB104 (35). It has recently been shown that *Abo* possesses  
20 at least two *alkB*-like genes, namely *alkB1* and *alkB2* (22, 3), both of which were induced by  
21 C<sub>14</sub> alkanes in *Abo* strain AP1 (3). However, knockout mutagenesis revealed that only *alkB1*  
22 is essential for the degradation of C<sub>6</sub> alkanes in *Abo* strain SK2 (22). Neither single *alkB1* or  
23 *alkB2* knockout mutants, nor a double mutant, exhibited significant growth deficiencies on  
24 C<sub>8</sub> to C<sub>16</sub> alkanes, so it was postulated that *Abo* specifies additional degradation systems for

1 alkane degradation. Consistent with this conclusion are our own transposon mutagenesis  
2 experiments with *Abo* strain SK2, which only yielded alkane-defective mutants with  
3 mutations mapping in genes specifying metabolic steps downstream of the initial alkane  
4 oxidation step, but not in *alkB1* and *alkB2* (data not shown). Thus, there is circumstantial,  
5 albeit negative, evidence for the existence of multiple systems for the primary oxidation of  
6 long chain alkanes.

7       Having in hand the recently finished genome sequence of *A. borkumensis* SK2  
8 (Schneiker et al., unpublished), we have sought evidence of potential additional alkane  
9 oxidation systems induced by growth of *Abo* on alkanes. We report here a proteomic study  
10 of differentially-regulated proteins in the membrane and the cytoplasmic fractions of *Abo*  
11 strain SK2, and propose from the results obtained the nature of the alkane oxidation systems  
12 available and of the metabolic adaptations to growth on alkanes.

13

## 14 **Materials and methods**

### 15 **Bacterial strain and growth conditions.**

16 *A. borkumensis* strain SK2 (DSM 11573) was grown on ONR7a medium at 30°C with  
17 agitation with either 2% (w/v) pyruvate or 1.5% hexadecane as carbon and energy source, as  
18 described earlier (50). In order to imitate the conditions of an oil spill in the environment  
19 (high carbon concentration and nitrogen limitation), we initially decided to create conditions  
20 of high C:N ratio (100:1) by having either 1.5% w/v hexadecane or 2% pyruvate. 1 ml of a  
21 pre-culture was used to inoculate 200 ml of the medium. Growth was monitored by  
22 measuring the OD<sub>600</sub>. The cells were harvested in the early exponential phase at an OD<sub>600</sub> of  
23 1.5 for the pyruvate culture and of 0.3 for the hexadecane culture.

1 **Preparation and two-dimensional gel electrophoresis of the cytoplasmic protein**  
2 **fraction.**

3 Cultures were harvested by centrifugation at 4°C at 8000g for 15 min, resuspended and  
4 washed twice in phosphate-buffered saline (40). The resulting pellet was stored at -20°C.  
5 For protein extraction, a cell pellet from a 200 ml culture was allowed to thaw on ice, and  
6 then 1 ml of rehydration buffer (4 % CHAPS, 30mM DTT, 20 mM Tris Base, 7 M urea, 2 M  
7 thiourea, 0.2 % IPG buffer, and one pellet of protease inhibitor cocktail (Complete™ Mini  
8 Boehringer, 20 ml<sup>-1</sup>) were added and the suspension sonicated on ice with a 3.5 mm  
9 sonication probe (Labsonic U; Braun, Melsungen, Germany) six times (91 W, repeating  
10 cycle of 0.6 s), with 30 s intervals between each cycle. The microfuge tubes were  
11 centrifuged to remove the cell debris. Benzonase® Nuclease (Novagen; 1/1000 dilution) and  
12 MgCl<sub>2</sub> (2 mM final conc.) were added to remove nucleic acids, and the tubes were incubated  
13 at 4°C for 1 h, after which the extracts were transferred to polycarbonate centrifuge tubes  
14 (Beckmann) and centrifuged for 45 min at 4°C (ca. 30000 x g, Rotor TLA100.3)  
15 (Beckmann ultracentrifuge). Two volumes of phenol (equilibrated with TE-buffer, pH 7.4)  
16 and two volumes of water were added to one volume of sample, vortexed vigorously,  
17 incubated on ice for 15 min and centrifuged. The aqueous phase was discarded without  
18 disturbing the white protein-containing lower phase. Two volumes of water were added and  
19 the procedure was repeated twice. Ice-cold acetone was added to precipitate proteins before  
20 the final centrifugation step. Protein pellets were washed twice with ice-cold acetone, air-  
21 dried, and resuspended in up to 500 µl rehydration buffer. The total protein concentration  
22 was determined by the Bradford method, using BSA as standard (4).

23 Two-dimensional gel electrophoresis was carried out as previously described (20).  
24 Briefly, 500 µg of protein in a total volume of 300 µl was subjected to isoelectric focusing in

1 IPG Ready Strips (17 cm) pH 3-6 or 4-7 (Bio-Rad, Munich, Germany). The gels were  
2 passively rehydrated for 2 h followed by an active rehydration step for 12 h at 50 V in  
3 rehydration buffer on a PROTEAN II Cell (Bio-Rad, Munich, Germany). Isoelectric  
4 focusing was performed at 5000 V for 150 Vh. For the second dimension the gels were  
5 soaked twice for 15 min in equilibration solution (6 M urea, 30 % glycerol, 2 % SDS, 50  
6 mM Tris base, pH 8.8), the first time with 2 % dithiotreitol (DTT) and the second time with  
7 2.5 % iodacetamide. The strips were then applied to 1.5 mm-thick gradient SDS-  
8 polyacrylamide (10 %-15 % w/v) gradient gels, which were subjected to electrophoresis at  
9 100 V in a IsoDalt system (Amersham Pharmacia Biotech, Uppsala, Sweden) overnight.  
10 Gels were stained with Colloidal Coomassie brilliant blue dye (CBB G-250) and proteins  
11 were cut out of gels, destained and prepared for matrix assisted laser desorption/ionization-  
12 time of flight (MALDI-TOF) analysis, according to Wissing *et al.* (49). The peptide mass  
13 fingerprints obtained were identified using an *A. borkumensis* SK2 protein database that was  
14 build up in conjunction with a genome sequencing study.

#### 15 **Preparation and two-dimensional gel electrophoresis of the membrane fraction.**

16 Cells from a 400 ml culture of Abo grown at 30°C on ONR7a medium were harvested (4500  
17 g x 20 min), resuspended in 10 mL 100 mM Tris-HCl (pH 7.0) buffer, incubated at 37 °C in  
18 a shaking water bath for 3 h, and sonicated on ice for 20 min at 50% power and a duty cycle  
19 of 5 in a Branson Sonifier. Membranes were pelleted by centrifugation at 30 000 g for 30  
20 min at 4°C, washed twice with the above buffer, and stored at -70°C, until use. Membrane  
21 proteins were isolated after resuspension of membranes in an equal volume of buffer,  
22 100 mM Tris-HCl (pH 7.0), containing 2% sodium-lauryl sarcosinate, 150 mM NaCl, and  
23 incubation at 37 °C for 1 h to facilitate inner membrane solubilization (16). Two volumes of  
24 equilibrated phenol (AppliChem GmbH, Darmstadt, Germany) were added to 1 volume of

1 sample, and the suspension was vigorously vortexed, incubated on ice for 10 min and  
2 centrifuged (16000 x g, 15 min, 4°C). The top aqueous phase was discarded, 2 volumes of  
3 distilled water were added, and the mixture was vortexed, incubated on ice for 10 min and  
4 centrifuged (4000 x g, 15 min, 4°C). The aqueous phase was discarded and the step  
5 repeated. Then 1 ml of ice-cold acetone was added, the tubes were inverted several times,  
6 incubated on ice for 10 min, and centrifuged (16000 x g, 15 min, 4°C). The liquid phase was  
7 discarded and the remaining pellet air-dried for 5-10 min. Pellets were suspended again in  
8 the solubilization solution and analysed by two-dimensional gel electrophoresis.

9       Two-dimensional gel electrophoresis was carried out as described previously (44),  
10 although with small modifications. Briefly, approximately 200 µg of protein was applied to  
11 24 cm pH 3-10NL IPG strips (ReadyStrip™, Bio-Rad, CA, USA) and fractionated by  
12 isoelectric focusing on a Protean IEF Cell (Bio-Rad) at a maximum voltage of 10,000 V for  
13 approximately 320 KVh according to the following program: 50 V, 100 Vh; 300 V, 800 Vh;  
14 600 V, 2,000 Vh; 2,500 V, 5,000 Vh; 7500 V, 30,000 Vh; 10000 V until the end of run. The  
15 strips were then loaded on 1.5 mm thick 10-15% gradient SDS-polyacrylamide gels and run  
16 overnight on a Hoefer DALT system (Amersham Biosciences). The gels were then fixed  
17 with 10% trichloroacetic acid, stained with Coomassie Brilliant Blue G250, and digitized  
18 images of stained gels were acquired by scanning. Protein spots were excised from  
19 preparative gels, *in situ* trypsin digestion (sequencing grade modified trypsin, Promega,  
20 Madison, WI, USA) and peptide extractions were performed as described previously (20).  
21 Peptide samples were eluted from ZipTips® U-C18 (Millipore, Bedford, MA, USA), using  
22 1.5 µl of saturated α-cyano-4-hydroxycinnamic acid (Sigma), and analysed by protein  
23 sequence using Quadrupole Time-of-Flight (Q-TOF) Mass Spectrometry.

24



## 1 **Prediction of putative promoters and operon structures**

2 Prediction of putative operons encompassing genes of interest was made on the basis of  
3 close vicinity (less than 50 bp) of similarly orientated genes encoding predicted functionally  
4 related proteins (19), and/or if a good putative promoter was found upstream of the first  
5 gene of the predicted operon. Putative  $\sigma^{70}$ -,  $\sigma^{54}$ -,  $\sigma^{38}$ -, and  $\sigma^{32}$ -dependent standard-type  
6 promoters of *Alcanivorax borkumensis* were identified by sequence homology to published  
7 consensus sequences (24, 26, 8, 15, correspondingly). We did not search for promoters of  
8 other types, as most of the putative operons of interest were actually preceded by one of  
9 these, which, in the context of predicting putative operon structure, was considered  
10 sufficient.

11

## 12 **Prediction of putative functions of novel proteins based on sequence homologies**

13 The putative functions of proteins with little or no homology to known proteins were  
14 predicted using tools currently available on the web. Firstly, the amino acid sequences of the  
15 identified proteins were obtained from the *Alcanivorax borkumensis* SK2 genome and  
16 subjected to a BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) homology search. Many of  
17 the proteins contained a conserved domain. Secondly, the amino acid sequences were also  
18 aligned to the *A. borkumensis* genome itself (BLAST to self). Thirdly, those genes/proteins  
19 with no functional domains were further analyzed by Pfam (<http://www.sanger.ac.uk/Pfam/>).  
20 Finally, the protein sequences were also checked for the presence of transmembrane  
21 domains using the program Tmpred (30).

22

23

24

## 1 **Results and Discussion**

### 2 **Proteins differentially expressed by cells grown on alkanes or pyruvate**

3           Since initial 2-D gel profiles of *Abo* proteins on IPG strips from pH 3-10 revealed  
4 that most of the cytoplasmic proteins focused in the range of pH 4-7 (data not shown), and  
5 the median value of pI for all the putative proteins from the draft genome sequence of  
6 *A.borkumensis* was calculated to be around 6.0, we concentrated on the pI range of pH 4-7.  
7 Differentially expressed (up- or down-regulated, or uniquely expressed, under either  
8 condition) cytoplasmic proteins influenced by alkane degradation by SK2 were identified by  
9 comparing two-dimensional gel electrophoresis images of proteins from cells in early  
10 stationary phase, growing with either pyruvate or hexadecane as sole source of carbon and  
11 energy, and Maldi-MS analysis of differentially-expressed proteins. Fig.1 shows Coomassie-  
12 stained 2-D gels of the cytoplasmic fraction of SK2 cells grown on either pyruvate (A) or  
13 hexadecane (B). Expression of most of the cytoplasmic proteins was found not to depend on  
14 the carbon source used, though twenty-five proteins consistently showed differential  
15 expression (Tables 1 and 2). Of these, seven appeared exclusively in gels of extracts from  
16 hexadecane-grown cells, namely outer membrane protein OprF (spot 2C),  
17 phosphoenolpyruvate synthase PspA-1 (spot 6C), malic enzyme MaeB (spot 8C), isocitrate  
18 lyase AceA (spot 9C), 50S ribosomal protein RplY (spot 16C), 30S ribosomal protein RpsB  
19 (spot 21C) and a putative acyl-CoA dehydrogenase (spot 22C), and eleven exclusively in  
20 gels of extracts from pyruvate-grown cells, namely outer membrane protein (spot 1C), outer  
21 membrane receptor FecA (spot 4C), fimbrial assembly protein precursor PilQ (spot 5C), 2,4-  
22 Diaminobutyrate (DABA) aminotransferase EctB (spot 7C), NADH-dependent isocitrate  
23 dehydrogenase Icd (spot 11C), conserved hypothetical protein (spot 17C), LysM domain  
24 protein (spot 18C), phosphate ABC transporter PstS (spots 19 and 20), acyl-CoA

1 dehydrogenase (spot 23C), and hypothetical protein (spot 24C). Three proteins showed  
2 significantly increased expression in alkane-grown cells, namely malate synthase GlcB (spot  
3 10C; 4x), fatty acid oxidation complex alpha subunit (spot 12C; 61x) and cytochrome P450  
4 (spot 25C; 1.9x), whilst 4 others were upregulated in pyruvate-grown cells: hypothetical  
5 protein (spot 3C; 2.7x), acetyl-CoA carboxylase AccA (spot 13C; 2.2x), acetyl-CoA  
6 carboxylase AccC (spot 14C; 1.9x), and long-fatty-acid CoA ligase FadD (spot 15; 2.7x).  
7 Identical Maldi spectra were obtained for the twin spots 17/51, 42/44 and 6/83.

8  
9 We employed a wider pI range (4-9) for the membrane fraction, to take into account  
10 the fact that most of the membrane proteins have rather extreme pIs. Obtaining clear 2-D  
11 images of membrane fractions from hexadecane-grown cells was problematic, probably due  
12 to the hydrophobic nature of the proteins expressed on hexadecane that affected isoelectric  
13 focusing. To circumvent the resulting difficulties in differentiating protein expression in  
14 pyruvate- and hexadecane-grown cells, we extracted all spots from both gels. Fig.2 shows  
15 Coomassie-stained 2-D gels of membrane proteins of *Abo* grown on pyruvate (A) or  
16 hexadecane (B), and Tables 1 and 2 list the differentially-expressed proteins. Thirty-eight  
17 proteins were detected exclusively in hexadecane grown-cells, namely alkane 1-  
18 monooxygenase AlkB (spot 1M), outer membrane lipoprotein LolB (spot 2M), rubredoxin  
19 AlkG (spot 4M), hypothetical proteins (spots 3M, 5, 7-9M), ABC transporters (spots 6M and  
20 83M), outer membrane protein OprG (spot 13M), the AlkS regulator of the *alkBIGHJ*  
21 operon (spot 14M), aldehyde dehydrogenase AlkH (spot 15), alcohol dehydrogenase AlkJ  
22 (spot 16M), medium-chain-fatty acid CoA ligase (spots 17M and 51M), succinate  
23 dehydrogenase SdhD (spot 22M), long-chain-fatty acid CoA ligase FadB (spot 23M), fatty  
24 acid oxidation complex FadB2 (spot 26M), (S)-2-hydroxy-fatty acid dehydrogenase RibD

1 (spot 27M), permease protein (spot 29M), cardiolipin synthase (spot 30M), conserved  
2 hypothetical protein (spot 32M), 3-oxoacyl-[acyl-carrier-protein] synthase (spot 34M), fatty  
3 acid desaturase (spots 42M and 44M), poly- $\beta$ -hydroxybutyrate polymerase PhaC (spot  
4 45M), putative metabolite transport transmembrane protein (spot 49M), ABC transporters  
5 (spots 63M and 83M), putative membrane protein (spot 64M), multidrug/solvent RND  
6 membrane fusion protein (spot 67M), putative membrane-associated metalloprotease (spot  
7 68M), putative lipoprotein (spot 73M), dihydroxy-acid dehydratase LlyD-1 (spot 75M),  
8 putative monooxygenase (spot 77M), alcohol dehydrogenase AlkJ-2 (spot 78M), nitrite  
9 extrusion protein NarK (spot 87M), and sodium solute transporter family protein (spot  
10 89M). Sixteen proteins were detected exclusively in pyruvate-grown cells, namely ectoin  
11 synthase EctC (spot 31M), outer membrane lipoprotein (spot 33M), inner membrane protein  
12 AmpE (spot 35M), putative membrane protein (spot 36M), putative outer membrane porin  
13 (spot 41M), outer membrane lipoprotein carrier protein LolA (spot 46M), ferric siderophore  
14 transport protein ExbD2 (spot 56M), hydrolase (spot 55M), membrane proteins (spots 61M  
15 and 65M), heavy metal RND efflux outer membrane protein CzcC (spot 62M), 2-  
16 oxoglutarate dehydrogenase LpdG (spot 74M), ABC transporter (spot 81M), dihydroxy-acid  
17 dehydratase IlvD2 (spot 82M), phosphate transporter (spot 85M), and oligopeptide ABC  
18 transporter (spot 88M). Identical Maldi spectra were obtained for the twin spots 19/20.

19

20 The combination of proteomic information obtained from the membrane and  
21 cytoplasmic fractions has yielded a rather comprehensive overview of the metabolic features  
22 of alkane-grown *Alcanivorax*, since enzymes involved in the primary attack of alkanes are  
23 mostly membrane-bound (48, 38, 42, 34), whereas subsequent metabolism of the metabolic  
24 products occurs in the cytoplasm. Differentially-expressed proteins fell into a number of

1 groups of functionally related proteins, often forming distinct gene clusters on the  
2 chromosome. These clusters were found to represent or include some 46 putative operons, as  
3 defined by common orientation of genes, a maximum of 50bp between successive genes  
4 (19), and the presence of putative promoters. Many of the membrane proteins up-regulated  
5 by growth on alkanes appeared to be related to metabolic pathways directly involved or  
6 closely linked to the metabolism of alkanes, namely the terminal oxidation of alkanes, fatty  
7 acid oxidation, and polyhydroxyalkanoate production, the latter representing a major  
8 pathway for carbon storage under conditions of excess carbon supply (Sabirova et al.,  
9 unpublished). Cytoplasmic responses to growth on alkanes were found to mostly concern the  
10 activity of intracellular carbon fluxes (glyoxylate bypass, fatty acid synthesis, and fatty acid  
11 oxidation). In addition we found a number of differentially-expressed conserved  
12 hypothetical proteins, and membrane proteins of unknown function, the potential functions  
13 of which we list in the Table 2, as far as they could be identified by the means of various  
14 sequence analysis tools.

15 In the following we analyze the principal metabolic routes that adapted to growth on  
16 alkanes.

17

### 18 **Terminal alkane oxidation**

19 Aerobic metabolism of alkanes generally proceeds through sequential oxidation of a  
20 terminal carbon, initiated by monooxygenases, which produce the alcohols, and followed by  
21 alcohol and aldehyde dehydrogenases, which produce the corresponding aldehydes and fatty  
22 acids, respectively. As indicated above, several monooxygenases and enzymes catalyzing  
23 subsequent oxidations were found uniquely in the membrane fraction of hexadecane-grown  
24 cells (Table 1). Among these was the entire set of enzymes of the *alkB1* operon, consisting

1 of ABO\_2707 encoding the AlkB1 alkane monooxygenase, ABO\_2708 encoding the AlkG  
2 rubredoxin, ABO\_2709 encoding the AlkH aldehyde dehydrogenase, and ABO\_2710  
3 encoding the AlkJ alcohol dehydrogenase (Fig.3A), described by van Beilen et al. (3) for *A.*  
4 *borkumensis* strain AP1. We also detected alkane-induced expression of the regulator AlkS  
5 (ABO\_2706), whose gene lies upstream of, and is divergently oriented from the *alkB1*  
6 operon, and which has been described to be the transcriptional activator of the *alkB* cluster  
7 in *Pseudomonas oleovorans* (13).

8 Other enzymes found to be alkane-induced, were cytochrome P<sub>450</sub> monooxygenase  
9 encoded by ABO\_0201 (and/or ABO\_2288, see below) and AlkJ2 alcohol dehydrogenase  
10 encoded by ABO\_0202, which form part of a putative operon comprising the genes  
11 encoding ferredoxin (ABO\_0200), cytochrome P<sub>450</sub>-1 (ABO\_0201), AlkJ2-alcohol  
12 dehydrogenase (ABO\_0202), and an oxidoreductase (ABO\_0203) (Fig.3C). The P<sub>450</sub>-1  
13 putative operon is closely linked to an AraC-like transcriptional regulator (ABO\_0199)  
14 reading into the opposite direction. The amino acid sequence of the AlkJ2-encoded alcohol  
15 dehydrogenase (ABO\_0202) shows strong homology to at least two other *A. borkumensis*  
16 alcohol dehydrogenases, one of which is AlkJ (ABO\_2710) of the *alkB1* operon. P<sub>450</sub>-1  
17 cytochrome encoded by ABO\_0201 is identical to a second P<sub>450</sub> cytochrome, P<sub>450</sub>-2,  
18 encoded by ABO\_2288 (Fig.3D), and also strongly homologous to a third P<sub>450</sub> cytochrome,  
19 P<sub>450</sub>-3, encoded by ABO\_2384 (Fig.3E). P<sub>450</sub> cytochromes belong to a superfamily of haem  
20 proteins found in all eukaryotes, as well as in most prokaryotes and archaea (37), which  
21 catalyze monooxygenation of a wide variety of organic molecules. The involvement of P<sub>450</sub>  
22 enzymes in alkane degradation has previously been shown for *Alcanivorax borkumensis*  
23 AP1, *Rhodococcus rhodochrous* ATCC 19067, *Acinetobacter calcoaceticus* EB 104,  
24 *Corynebacterium sp.* and some hydrocarbon-degrading yeasts (2, 6, 35, 32). Fig. 1 revealed

1 expression of either P<sub>450-1</sub> and/or P<sub>450-2</sub>, in cells grown on either alkane or pyruvate, but at  
2 higher levels in alkane-grown cells. Whereas P<sub>450-1</sub>, although not apparent on 2-D gels, is  
3 presumably up-regulated in cells grown on alkanes, since its gene is in the same operon as  
4 that of the up-regulated *alkJ2* gene, this is probably not the case for P<sub>450-2</sub> and P<sub>450-3</sub>. *In*  
5 *silico* comparison of the regions upstream of ABO\_0201 (P<sub>450-1</sub>) and ABO\_2288 (P<sub>450-2</sub>) to  
6 promoter consensus sequences of other bacteria, revealed that these two genes are preceded  
7 by different putative promoter sequences and are thus likely to be differently regulated  
8 (Fig.3C and 3D). While ABO\_0201 is likely to be induced by alkanes, ABO\_2288 is  
9 probably constitutively expressed. It is not yet clear which role, other than the primary  
10 attack of alkanes, P<sub>450S</sub> may play in *Alcanivorax*, such as in cells growing on pyruvate.  
11 However, there is evidence that P<sub>450</sub> in *Bacillus subtilis* is involved in supplying pimelic acid  
12 equivalents for the synthesis of biotin (10), a cofactor of the principal enzymes of fatty acid  
13 biosynthesis.

14

15 We have also detected alkane-induced expression of a putative monooxygenase  
16 encoded by ABO\_0190 (Fig. 3F). *In silico* analysis identified this monooxygenase as a  
17 flavin-binding monooxygenase (FMO), belonging to a family (pFam) of xenobiotic-  
18 metabolising enzymes, and with 52% identity/68% similarity to cyclohexanone  
19 monooxygenase of *Ralstonia eutropha* strain JMP134), an enzyme that mediates oxidation  
20 of cyclohexanone, the second step in the metabolism of cyclohexane. Inspection of the  
21 *Alcanivorax* genomic context of ABO\_0190 revealed a putative operon of 4 genes encoding  
22 caprolactone hydrolase (ABO\_0191), cyclohexanone monooxygenase (ABO\_0190),  
23 cyclohexanol dehydrogenase (ABO\_0189) and metal-dependent hydrolase (ABO\_0188) that  
24 are probably involved in the metabolism of cycloalkanes, and convert cyclohexanol to 6-

1 hydroxyhexanoic acid. We did not find a gene encoding cyclohexane monooxygenase in the  
2 *Abo* genome, and therefore suspect that another enzyme mediates the initial attack of cyclic  
3 alkanes in *Alcanivorax*, and that this enzyme may in fact be alkane hydroxylase, encoded by  
4 either *alkB1*, or by *alkB2*, since Fujii et al. (17) recently showed oxidation of cycloalkanes  
5 by the alkane hydroxylase system (comprising alkane 1-monooxygenase AlkB, rubredoxin  
6 AlkG, and rubredoxin AlkT) of *Gordonia* sp. TF6. The assumption that initial oxidation of  
7 linear alkanes and cyclohexanes by *Abo* is mediated by the same AlkB1/AlkB2  
8 monooxygenase is consistent with (i) the inability of *Abo* to utilize cyclohexane as sole  
9 source of carbon and energy (data not shown), but to co-metabolize it well when grown on  
10 crude oil (Yakimov et al., unpublished), and (ii) the apparent coinduction by hexadecane of  
11 both AlkB1 and the predicted cyclohexane-degrading operon containing the up-regulated  
12 putative cyclohexanone monooxygenase ABO\_0190 (also reflected in some marked  
13 similarities of the respective operon upstream regions which both exhibit putative  $\sigma^{70}$   
14 promoters as well as some perfectly conserved sequence motives of a likely regulatory  
15 function (data not shown). These tentative conclusions await experimental confirmation.

16

17

## 18 **Fatty acids, lipids and membranes**

19 Fatty acids produced during growth on alkanes are transformed into CoA-activated  
20 fatty acids, which are further degraded via  $\beta$ -oxidation. As it was expected, alkane  
21 degradation by *Abo* is associated with increased expression of enzymes of the  $\beta$ -oxidation  
22 pathway. Interestingly, two entire sets of  $\beta$ -oxidation enzymes were induced by growth on  
23 alkanes, namely ABO\_0184 and ABO\_2748 which encode fatty acid CoA ligases  
24 (synthetases), ABO\_2102 and Abo\_0988, which encode acyl-CoA dehydrogenases, and



1 ABO\_1652 and ABO\_1566, which encode bifunctional components of the  $\beta$ -oxidation  
2 multifunctional enzyme complex and that possess both 3-hydroxyacyl-CoA dehydrogenase  
3 and enoyl-CoA hydratase activities. ABO\_1652 and ABO\_1566 encode components of two  
4 different enzyme complexes involved in alkane-induced fatty acid oxidation, one of which,  
5 ABO\_1652, corresponding to *fadB2* of the *fadAB2* operon, is exclusively expressed in cells  
6 grown on hexadecane, while the latter, ABO\_1566, corresponding to *fadB* of the *fadAB*  
7 operon, though clearly up-regulated in presence of alkanes, is also expressed in cells grown  
8 on pyruvate.

9         Apart from enhanced expression of genes mediating fatty acid degradation, we also  
10 detected a significant alkane-induced increase of expression of fatty acid biosynthesis  
11 determinants, namely the *fabAB* operon ABO\_0835 and ABO\_0834, encoding  $\beta$ -  
12 hydroxyacyl-acyl carrier protein dehydratase (FabA) and  $\beta$ -ketoacyl-acyl carrier protein  
13 synthase I (FabB), and ABO\_1520, which encodes a second FabB homologue. The Fab  
14 enzymes of *E. coli* (41, 9) and *Pseudomonas aeruginosa* (25) have been shown to be  
15 specifically required for the synthesis of unsaturated fatty acids. It is known that growth of  
16 *Pseudomonas oleovorans* GPo1 on alkanes results in substantial accumulation of AlkB  
17 alkane hydroxylase protein in the inner membrane (14), that would provoke perturbation of  
18 membrane structure and physical characteristics, if not compensated by an increase in lipids  
19 with unsaturated fatty acids (7). The up-regulation of enzymes involved in the synthesis of  
20 unsaturated fatty acids is presumably associated with the need to maintain membrane  
21 fluidity and integrity in step with increasing AlkB protein-induced perturbations.

22         In contrast, we observed alkane-induced down-regulation of cytoplasmic proteins  
23 acetyl-CoA carboxylase *AccA*, encoded by ABO\_1159, and acetyl-CoA carboxylase *AccC*,  
24 encoded by ABO\_2010, components of a multicomponent system catalyzing the first step in

1 the synthesis of fatty acids, namely the production of malonyl-CoA from acetyl-CoA, also  
2 involved in fatty acid biosynthesis. This is consistent with a reduced requirement for malate  
3 in alkane-grown cells, which is produced in abundance as a result of increased activity of the  
4 glyoxylate bypass (see below).

5 Alkane-induced changes in the composition of the cellular fatty acid pool are  
6 indicative of concomitant changes in membrane lipid composition (and indeed membrane  
7 composition). In this regard, we observed increased expression of ABO\_1816, which  
8 encodes cardiolipin synthase (Cls) in alkane-grown cells. Cardiolipin has been shown to  
9 have the potential to form non-bilayer structures, which introduce discontinuities into lipid  
10 bilayers and thus to facilitate dynamic changes in membrane structures, such as membrane  
11 fusion events (e.g. the formation of adhesion sites between the outer and inner membranes,  
12 during cell division; 12), but also to activate membrane-bound enzymes, like AlkB (27, 36).  
13 Importantly, increased cardiolipin synthesis may also constitute a protective membrane  
14 adaptation to decrease membrane permeability to organic solvents, as it has been shown for  
15 pseudomonads (47).

16 Another alkane-induced change related to lipid metabolism that we observed was the  
17 up-regulation of genes coding for the lipoprotein releasing proteins (Lol), which target and  
18 anchor lipoproteins to the periplasmic surface of either the inner or the outer membrane,  
19 depending on the sorting signal (46). The Lol system consists of an ATP-binding cassette  
20 transporter, encoded by *lolCDE*, which transports outer membrane-specific lipoproteins  
21 across the inner membrane into the periplasmic space, where they are released. An inter-  
22 membrane shuttle complex then forms between the released lipoproteins and the LolA  
23 periplasmic chaperone, which then associates with the LolB outer membrane-located  
24 permease to complete the targeting process (46). Expression of ABO\_1049 and ABO\_1050,

1 encoding the LolCDE transporter, and Abo\_0520, encoding the LolB permease, was up-  
2 regulated in alkane-grown cells. Surprisingly, expression of ABO\_1291, which encodes the  
3 LolA periplasmic chaperone, was only detected in cells grown on pyruvate. This finding  
4 may, however, be misleading as the LolA chaperone may be tightly complexed with its  
5 target lipoproteins, and thus in a form not readily resolved by proteomics. Alkane-induced  
6 expression of the Lol system in *Alcanivorax* might reflect an increased need to release  
7 lipoproteins, since some of these have been shown to possess emulsifying properties that  
8 increase the surface area and hence enhance the bioavailability of hydrophobic substrates  
9 (31, 33, 51).

10

### 11 **Glyoxylate bypass and gluconeogenesis**

12 During growth on alkanes as sole carbon source, bacteria must generate all cellular  
13 precursor metabolites from acetyl-CoA, the main intermediate formed during alkane  
14 degradation via  $\beta$ -oxidation of fatty acids. One mechanism to do this is the short circuiting  
15 of the citric acid cycle, through activation of the glyoxylate bypass, which routes acetyl-CoA  
16 to the key 3-carbon metabolite phosphoenolpyruvate (PEP), via isocitrate, glyoxylate and  
17 malate, by means of isocitrate lyase and malate synthase, thereby avoiding the CO<sub>2</sub>-  
18 releasing steps of the cycle (Fig.4). A significant feature of alkane-grown cells is the up-  
19 regulation of ABO\_2741, encoding isocitrate lyase AceA, and ABO\_1267, encoding malate  
20 synthase GlcB, and down-regulation of enzymes mediating CO<sub>2</sub>-releasing steps of the TCA  
21 cycle short circuited by the glyoxylate shunt, including ABO\_1281, encoding isocitrate  
22 dehydrogenase Icd, and ABO\_1494, encoding 2-oxoglutarate dehydrogenase LpdG. We also  
23 suspect down-regulation of another enzyme indicative of the complete TCA, namely  
24 succinyl-CoA synthetase SucC, encoded by ABO\_1493, as, according to our *in silico*

1 analysis, this gene is located in the same putative operon as ABO\_1494. Another enzyme of  
2 the TCA cycle needed for the glyoxylate bypass, namely succinate dehydrogenase SdhD  
3 (ABO\_1499), was also found to be up-regulated in alkane-grown cells (Fig.4).

4 The hypothesis that all biosynthetic precursors come from acetyl-CoA in alkane-  
5 grown cells is also consistent with the finding that enzymes involved in gluconeogenesis,  
6 namely malic enzyme MaeB (ABO\_2239) and phosphoenolpyruvate synthase PspA-1  
7 (ABO\_1427), were up-regulated (Fig.4). Thus, the key metabolic intermediate in alkane-  
8 grown cells is malate, formed through channeling acetyl-CoA into the glyoxylate bypass.

#### 9 10 **Polyhydroxyalkanoate biosynthesis**

11 For carbon-limited microbes, an increase in carbon allows an increase in growth rate  
12 until another growth limitation is reached. The appearance of alkanes in oligotrophic  
13 environments like most marine habitats allows *Alcanivorax* to “bloom” initially until  
14 nitrogen limitation is experienced. Under conditions of high C:N ratios, many microbes  
15 synthesize carbon storage materials, like polyhydroxyalkanoates (PHAs). ABO\_1418, one  
16 of two *A. borkumensis phaC* PHA synthase genes was solely expressed in alkane-grown  
17 cells, whereas another, ABO\_2214, was not expressed at detectable levels in such cells.  
18 Since PHA is also produced at high C:N ratios in cells grown on non-alkane substrates  
19 (Sabirova et al., unpublished), it seems that Abo produces either of two distinct PhaC PHA  
20 synthases in response to different growth substrates, probably having different substrate  
21 specificities that reflect distinct metabolites produced from the different growth substrates.

## 1 **Cofactor synthesis**

2 Alkane metabolism in *Alcanivorax* involves a number of enzymes, in particular  
3 monooxygenases, containing cofactors as active groups. ABO\_217, encoding RibD ((S)-2-  
4 hydroxy-fatty-acid dehydrogenase), a key enzyme of the riboflavin synthesis pathway, is up-  
5 regulated in alkane-grown cells. Riboflavin is the precursor of flavin mononucleotide  
6 (FMN) and flavin adenine dinucleotide (FAD), cofactors of enzymes involved in reduction  
7 processes and of electron transport proteins, such as dehydrogenases, oxidases, and  
8 monooxygenases. Specifically, riboflavins are cofactors of the flavin-binding  
9 monooxygenase ABO\_0190 and of the FMN-binding domains of cytochromes P<sub>450</sub> encoded  
10 by ABO\_0201 and ABO\_2288. On the other hand, ABO\_1963, encoding lipoil-(acyl-carrier  
11 protein)-protein-*n*-lipoyltransferase, an enzyme involved in biotin biosynthesis, is down-  
12 regulated in alkane-grown cells. Down-regulation of biotin biosynthesis is consistent with  
13 the alkane-induced repression of the *accA* and *accC* genes, encoding key enzymes of the  
14 fatty acid biosynthetic route, in which biotin serves as a cofactor.

15

16

## 17 **Conclusions**

18 The data presented here strongly suggest that alkane degradation in *Alcanivorax* proceeds  
19 via several routes of terminal oxidation, involving AlkB hydroxylases, a putative flavin-  
20 binding monooxygenase and P<sub>450</sub> cytochrome(s). Since certain individual *n*-alkanes and  
21 cycloalkanes are not growth substrates, but are metabolized when present in hydrocarbon  
22 mixtures, they may not be inducers of the initial monooxygenases and require other alkanes  
23 to induce the appropriate catabolic enzymes. Alkane degradation strongly modifies  
24 metabolism, especially intracellular carbon fluxes and membrane lipid composition. The

1 glyoxylate bypass and gluconeogenesis routes induced by alkanes adapt the cell to produce  
2 key cellular precursor metabolites directly from the fatty acids produced by alkane  
3 oxidation. Despite the fact that, as would be expected, fatty acid synthesis is downregulated  
4 during growth on alkanes, there is an upregulation of the synthesis of unsaturated fatty acids,  
5 presumably reflecting a need for resulting in changes in the composition of membrane  
6 lipids. These results provide new insights into the metabolic adaptations needed for growth  
7 on alkanes and into the genomic basis of the hydrocarbonoclastic lifestyle. Since the 2-D  
8 maps have revealed a number of upregulated proteins with unknown functions in alkane  
9 metabolism, their study will surely bring further important insights into this unique lifestyle.

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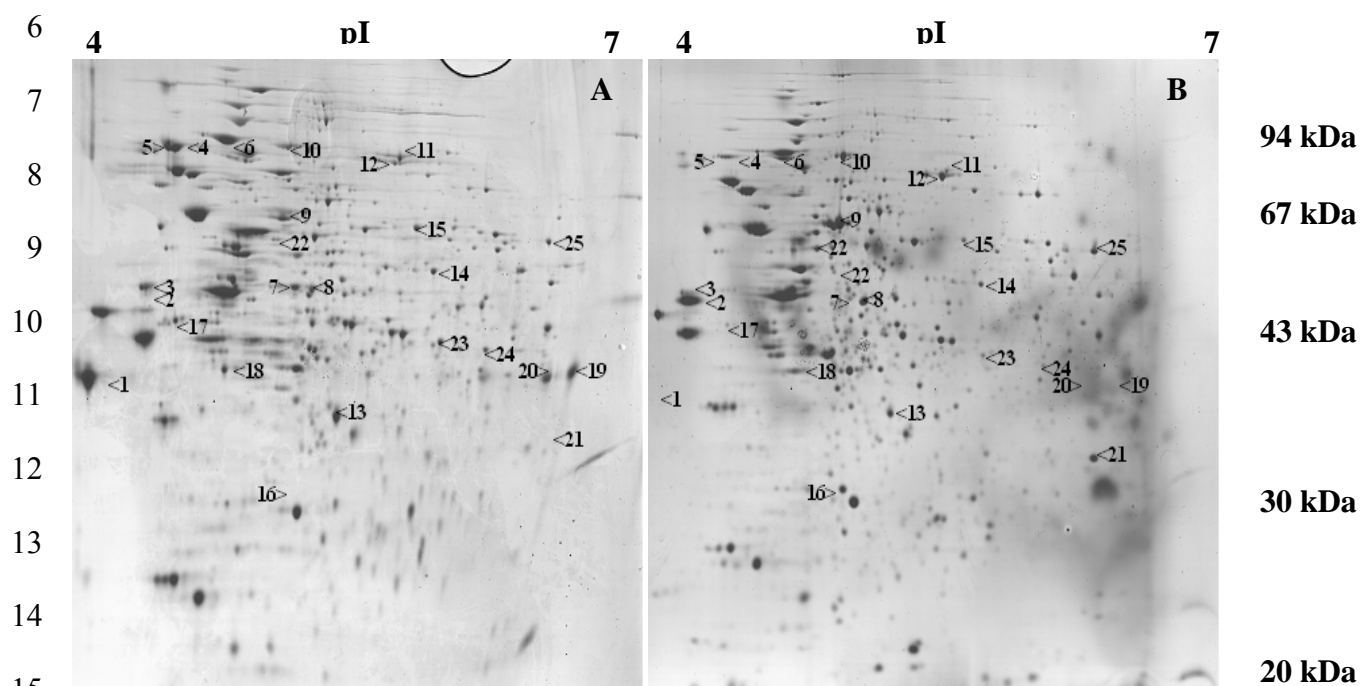
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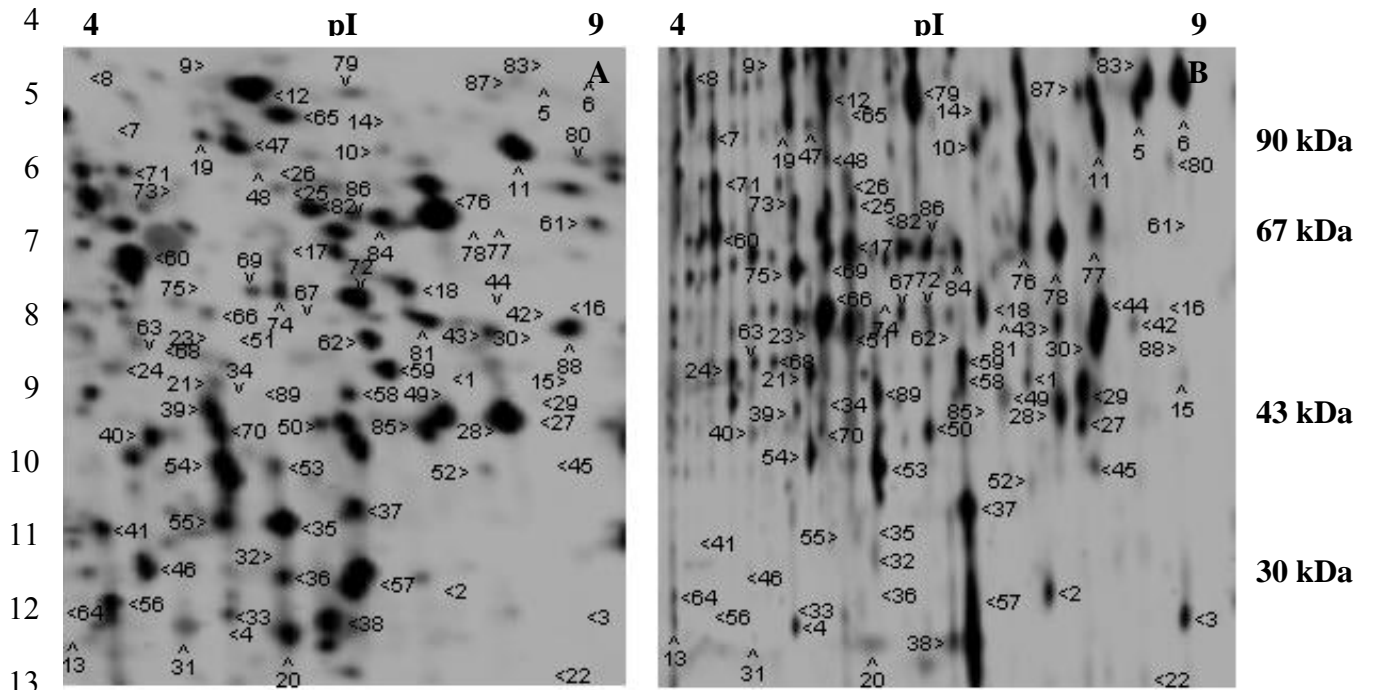
## Figures and Tables

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3 **Fig.1.** 2-D map of the cytoplasmic proteins of *Alcanivorax borkumensis* SK2. Cells were  
4 grown on either pyruvate (A) or hexadecane (B). Isoelectric focusing was performed using an  
5 IPG-strip of pH 4-7. Proteins showing reproducible differential expression are numbered.



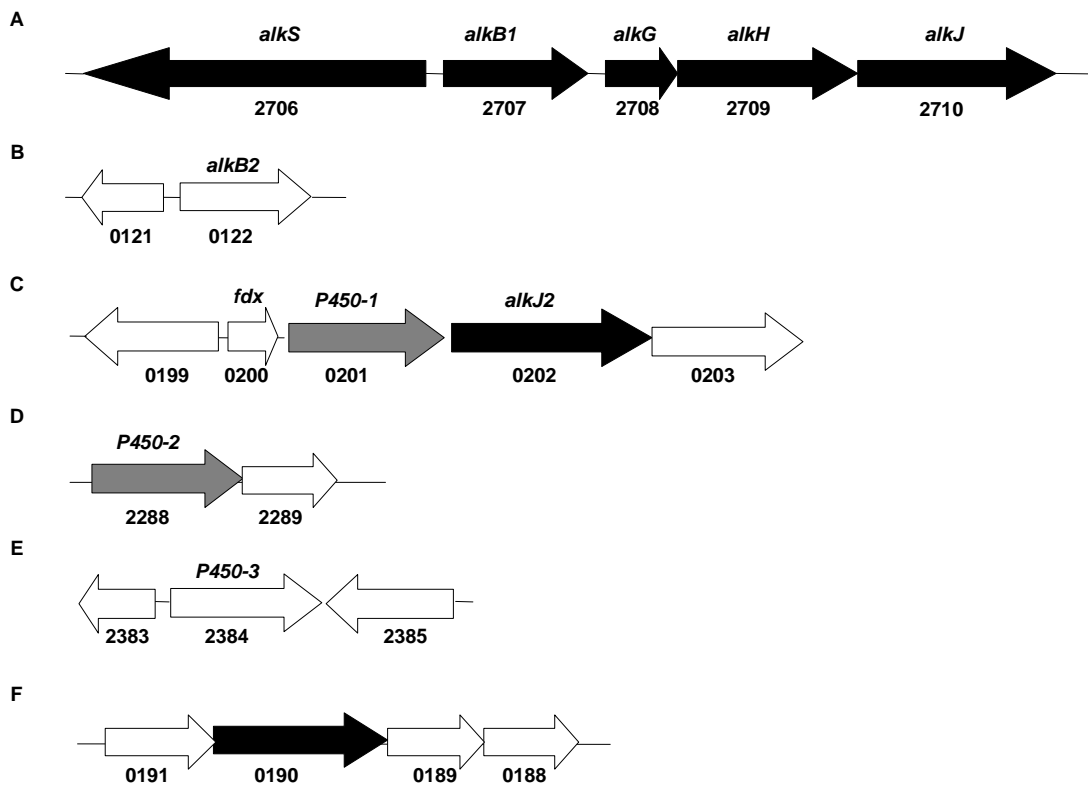
1 **Fig.2.** 2-D map of the membrane proteins of *Alcanivorax borkumensis* SK2. Cells were grown  
 2 on either pyruvate (A) or hexadecane (B). Isoelectric focusing was performed using an IPG  
 3 strip of pH 4-9. All membrane proteins identified are numbered.



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1 **Fig.3.** Schematic representation of genomic regions containing genes encoding  
 2 monooxygenases and enzymes presumed to be involved in terminal oxidation of alkanes.  
 3 Genes up-regulated in alkane-grown cells are colored in black; white-colored ORFs with  
 4 black frame show homologous genes in the *Abo* genome that seem not to be up-regulated;  
 5 grey-colored ORFs encode the p<sub>450</sub>-1 and p<sub>450</sub>-2 enzymes.

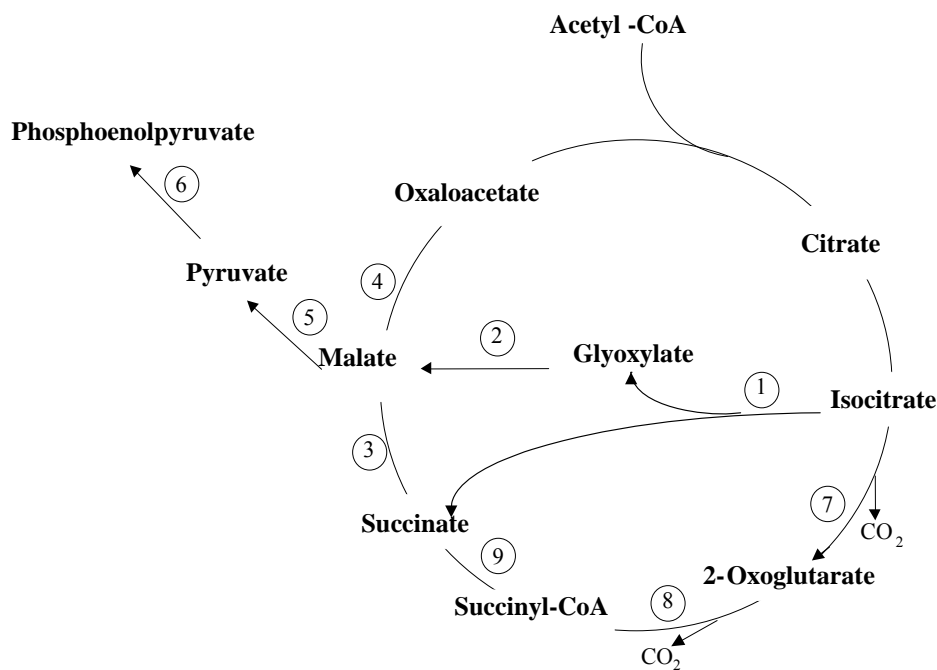
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1 **Fig.4. Increased exploitation of the glyoxylate bypass of the TCA cycle in alkane-grown**  
 2 **cells of *Alcanivorax*.** The glyoxylate bypass is carried out by isocitrate lyase (1) and malate  
 3 synthase (2). Succinate produced via glyoxylate bypass is converted to malate by succinate  
 4 dehydrogenase (3). Malate is converted to either oxaloacetate by malate dehydrogenase (4),  
 5 or is used by malic enzyme (5) in gluconeogenesis to produce pyruvate. Pyruvate is then  
 6 converted by phosphoenolpyruvate synthase (6) to produce phosphoenolpyruvate. The  
 7 incomplete TCA cycle is associated with the alkane-induced down-regulation of isocitrate  
 8 dehydrogenase (7), 2-oxoglutarate dehydrogenase (8), and succinyl-CoA synthetase (9).



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1 **TABLE 1.** Differentially expressed proteins derived from both membrane and cytoplasmic protein fractions of *A.borkumensis* SK2 grown on  
 2 either hexadecane or pyruvate as carbon source.

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Spot no.	pI	MW	Gene function or functional category <sup>1</sup>	Gene number <sup>1</sup>	Differential abundance <sup>2</sup>	Putative operon <sup>3</sup>
<b>Terminal oxidation of alkanes</b>						
14M	6.4	98.1	Regulator of the alkB1GHJ operon (AlkS)	ABO_2706	H	1
1M	6.5	46.5	Alkane 1-monooxygenase (AlkB)	ABO_2707	H	1
4M	4.5	18.7	Rubredoxin (AlkG)	ABO_2708	H	1
15M	9.5	52.8	Aldehyde dehydrogenase (AlkH)	ABO_2709	H	1
16M	9.3	58.3	Alcohol dehydrogenase (AlkJ)	ABO_2710	H	1
77M	9.5	57.3	Monooxygenase (putative)	ABO_0190	H	2
78M	8.4	60.6	Alcohol dehydrogenase (AlkJ-2)	ABO_0202	H	3
25C	6.2	53.5	Cytochrome P450-1	ABO_0201	<sup>4</sup>	3
25C	6.2	53.5	Cytochrome P450-2	ABO_2288	<sup>4</sup>	-
<b>Fatty acid oxidation</b>						
23M	4.6	56.3	Long-chain-fatty-acid-CoA ligase (FadB)	ABO_0184	H	4
15C	5.4	62.1	Long-fatty-acid CoA ligase, putative (FadD)	ABO_0367	2.7 down	5

17M 51M	5.0	59.8	Medium-chain-fatty-acid CoA ligase (AlkK)	ABO_2748	H	-
22C	4.8	64.5	Acyl-CoA dehydrogenase (putative)	ABO_2102	H	-
23C	5.6	42.5	Acyl-CoA dehydrogenase	ABO_0988	7.2 up	6
26M	5.3	77.3	Fatty acid oxidation complex (FadB2)	ABO_1652	H	7
12C 25M	5.5	78.0	Fatty oxidation complex alpha subunit (FadB)	ABO_1566	61 up	8
<b>Fatty acid and phospholipid biosynthesis</b>						
13C	5.2	35.7	Acetyl-CoA carboxylase, carboxyl transferase, alpha subunit (AccA)	ABO_1159	2.2 down	-
14C	5.6	48.9	Acetyl-CoA carboxylase, biotin carboxylase (AccC)	ABO_2010	1.9 down	9
34M	5.0	42.7	3-Oxoacyl-[acyl-carrier-protein] synthase (FabB)	ABO_0834	H	10
53M	5.2	41.3	3-Oxoacyl-[acyl-carrier-protein] synthase (FabB)	ABO_1520	6.4 up	-
54M	4.9	42.1	3-oxoacyl-(acyl-carrier-protein) synthase (FabF)	ABO_1071	10.0 down	11
42M 44M	9.6	45.3	Fatty acid desaturase (putative)	ABO_2585	H	-
52M	8.9	36.4	Sterol desaturase family protein	ABO_0114	2.3 down	-
30M	9.1	54.1	Cardiolipin synthase (Cls)	ABO_1816	H	-

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**Amino acid biosynthesis**

75M	4.9	58.5	Dihydroxy-acid dehydratase (IlvD-1)	ABO_0180	H	4
82M	5.2	70.9	Dihydroxy-acid dehydratase (IlvD-2)	ABO_2312	P	-

**TCA, respiratory chain, glyoxylate bypass, and gluconeogenesis**

11C	5.5	82.6	Isocitrate dehydrogenase, NADH-dependent (Icd)	ABO_1281	2.0 down	12
74M	4.8	51.2	2-Oxoglutarate dehydrogenase (LpdG)	ABO_1494	P	13
55M	5.1	32.0	Hydrolase	ABO_1541	P	14
9C	4.9	59.1	Isocitrate lyase (AceA)	ABO_2741	36 up	-
10C	4.9	78.7	Malate synthase (GlcB)	ABO_1267	6.1 up	-
6C	4.7	86.9	Phosphoenolpyruvate synthase (PpsA-1)	ABO_1427	H	-
8C	5.0	45.8	Malic enzyme (MaeB)	ABO_2239	3.1 up	15
22M	8.8	13.2	Succinate dehydrogenase, hydrophobic membrane anchor protein (SdhD)	ABO_1499	H	16
76M	7.8	59.0	Oxidoreductase, GMC family	ABO_0187	5.6 up	4

**Polyhydroxyalkanoate production**

45M	9.7	41.9	Poly-beta-hydroxybutyrate polymerase (PhaC)	ABO_1418	H	-
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**Osmoprotection**

31M	4.8	14.8	Ectoin synthase (EctC)	ABO_2152	P	17
7C	5.0	48.4	DABA aminotransferase (EctB)	ABO_2151	P	17

**Cofactor synthesis**

27M	8.7	40.4	(S)-2-Hydroxy-fatty-acid dehydrogenase ribD,	ABO_2174	H	18
38M	6.2	27.3	Lipoil-(acyl-carrier protein)-protein-n-lipoyltransferase (LipB)	ABO_1963	26 down	19

**Pili formation**

5C	4.4	78.1	Fimbrial assembly protein precursor (PilQ)	ABO_2233	46 down	20
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**Information processing and regulation**

21C	6.3	28.2	30S Ribosomal protein S2 (RpsB)	ABO_1143	H	21
16C	4.9	23.7	50S Ribosomal protein L25 (RplY)	ABO_0517	H	22
79M	6.5	91.6	Sensor histidine kinase	ABO_0442	14.0 up	-

**Transport proteins**

6, 83M	9.0	87.1	ABC transporter, permease protein (putative)	ABO_1402	H	23
21M	4.8	47.1	ABC export system, membrane fusion protein	ABO_0248	7.2 up	24
63M	4.3	49.7	ABC export system, outer membrane protein	ABO_0250	H	24

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81M	7.6	41.0	ABC transporter, ATP-binding protein, pernease (putative)	ABO_1847	P	25
84M	6.5	67.6	Oligopeptide ABC transporter, periplasmic peptide-binding protein	ABO_1219	5.2 down	26
88M	8.6	69.6	Oligopeptide ABC transporter, peripasmic peptide-binding protein	Abo_1220	P	26
19C 20C	9.2	37.3	Phosphate ABC transporter periplasmic binding protein (PstS)	ABO_2685	P	27
85M	8.7	44.3	Phosphate transporter (putative)	ABO_2305	P	-
89M	5.9	56.4	Sodium solute transporter family protein	ABO_1913	H	-
87M	9.7	95.9	Nitrite extrusion protein (NarK)	ABO_0547	H	28
49M	5.8	46.8	Metabolite transport transmembrane protein, putative	ABO_2038	H	-
24M	4.0	44.5	Long-chain fatty acid transporter, putative	ABO_0572	20 up	-
69M	5.0	62.5	Heavy metal RND efflux membrane fusion protein,CzcB family (CzcB2)	ABO_1357	3.1 up	29
62M	6.2	48.1	Heavy metal RND efflux outer membrane protein, CzcC family (CzcB1)	ABO_1358	P	29
59M	6.5	44.5	Heavy metal RND efflux membrane fusion protein, CZsB family (CzcB3)	ABO_1382	4.1 down	30

39M	4.6	40.7	Outer membrane polysaccharide export protein precursor (Wza)	ABO_0905	4.2 down	31
41M	3.9	36.1	Outer membrane porin (putative)	ABO_1621	P	-
4C 48M	4.4	80.2	FecA-like outer membrane receptor (FecA)	ABO_0721	43 down 2.5 up	-
56M	4.4	14.1	Ferric siderophore transport system, inner membrane protein E (ExbD2)	ABO_1968	P	32
46M	4.2	23.0	Outer membrane lipoprotein carrier protein (LolA)	ABO_1291	P	33
2M	8.8	21.8	Outer membrane lipoprotein (LolB)	ABO_0520	H	34
29M	8.6	45.6	Lipoprotein releasing system, permease protein, putative	ABO_1049	H	35
67M	5.6	47.8	multidrug/solvent RND membrane fusion protein (putative)	ABO_0965	H	36
68M	4.3	46.5	Membrane-associated zink metalloprotease (putative)	ABO_1150	H	37

1 <sup>1</sup> - Gene numbers, gene function or functional category are presented according to the annotated genome (Schneiker et al., unpublished ); <sup>2</sup> - H means  
2 that the protein is solely expressed on hexadecane; P means that the protein is solely expressed on pyruvate; “down” means that the protein is down-  
3 expressed on hexadecane; “up” means the protein is up-expressed on hexadecane; <sup>3</sup> – Putative operons demonstrated in this table are at least two  
4 consecutive genes encoding co-expressed functionally related proteins, closely associated on the chromosome and transcribed from their own putative  
5 promoter, identified by in silico analysis; <sup>4</sup> - For the expression pattern of the p450 cytochromes please refer to the text; <sup>TM</sup>- transmembrane domains  
6 (based on the TMHMM (30), a transmembrane helices prediction method based on a hidden Markov model (HMM).

**TABLE 2.** Differentially expressed proteins of uncertain or unknown function

Spot no.	pI	MW	Gene function or functional category <sup>1</sup>	Gene number <sup>1</sup>	Differential abundance <sup>2</sup>	Putative operon <sup>3</sup>	Putative function based on sequence analysis tools
20M	4.9	17.3	Putative membrane protein	ABO_0097	31 down	-	Membrane protein implicated in regulation of membrane protease activity.
18C	6.0	49.7	LysM domain protein	ABO_0132	4.1 down	-	Lysin domain, found in enzymes involved in bacterial cell wall degradation.
28M	7.1	42.2	Hypothetical protein	ABO_0154	2.8 down	-	No putative domains have been detected.
3M	9.4	23.7	Hypothetical protein	ABO_0160	H	-	No putative domains have been detected.
3C	4.2	48.7	Hypothetical protein	ABO_0193	2.7 down	38	similar to long-chain fatty acid transport protein of <i>Marinobacter aquaeolei</i> VT8.
33M	4.9	15.2	Outer membrane lipoprotein (OmlA)	ABO_0308	P	-	OmlA gene encodes a novel lipoprotein in <i>Ps. aeruginosa</i> . As in <i>Pseudomonas</i> the gene omlA is immediately upstream of divergently transcribed fur (ferric uptake regulator).
64M	3.7	19.9	Membrane protein (putative)	ABO_0443	H	-	ompA-like transmembrane domain is present in a number of different outer membrane proteins of several gram-negative bacteria.
73M	4.8	69.4	Lipoprotein ( putative)	ABO_0586	H	39	Homologous to LppC gene. A part of a putative operon with gmhA gene (Abo_556) encoding



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							for phosphoheptose isomerase, required for lipooligosaccharide biosynthesis in other bacteria (5).
35M	6.2	31.9	Inner membrane protein (AmpE)	ABO_0621	P	40	The AmpE gene encodes a transmembrane protein with unknown function. Upstream-located AmpD gene (Abo_587) encodes a cytosolic N-acetyl-anhydromuramyl-L-alanine amidase that participates in the intracellular recycling of peptidoglycan fragments.
65M	5.9	87.7	Membrane protein (putative)	ABO_0666	P	41	Predicted exporter of the RND superfamily
40M 2C	4.1	42.0	Outer membrane protein (OprF)	ABO_0822	55 down H	-	
61M	9.2	63.1	Membrane protein	ABO_0929	P	42	Putative sodium-sulphate transporter. TM
66M	4.6	51.2	Membrane protein (putative)	ABO_0963	15.4 up	36	Outer membrane efflux protein. Forms trimeric channels that allow export of a variety of substrates in Gram negative bacteria.
7M	3.7	84.8	Conserved hypothetical protein	ABO_0997	H	-	No putative domains have been detected. TM
47M	4.9	84.6	Membrane protein (putative)	ABO_1242	12 up	-	His Kinase A (phosphoacceptor) domain.

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36M	5.5	29.3	Membrane protein (putative)	ABO_1323	P	43	PEP:sugar phosphotransferase system, putatively involved in uptake of pyruvate in <i>Alcanivorax</i> . A part of a putative operon with the upstream gene (Abo_1249) encoding conserved hypothetical protein with pyruvate phosphate dikinase domain.
5M	8.5	101.8	Conserved hypothetical protein	ABO_1398	H	-	No putative domains have been detected. TM.
8M	3.5	108.8	Conserved hypothetical protein	ABO_1464	H	44	Tfp pilus assembly protein FimV. A part of an operon with the downstream truA gene in <i>Ps. aeruginosa</i> (1). The fimV gene is required for twitching motility while the truA gene is required for the type III secretory gene expression.
9M	4.8	105.3	Conserved hypothetical protein	ABO_1589	H	45	Family of proteins of unknown function.
32M	6.9	28.1	Conserved hypothetical protein	ABO_1588	H	45	Predicted spermidine synthase with an N-terminal membrane domain.
1C	3.9	36.1	Outer membrane protein	ABO_1621	P	-	Porins
24C	5.9	41.8	Hypothetical protein	ABO_1657	4.1 down	46	No putative domains have been detected.
12M	4.5	95.7	Conserved hypothetical protein	ABO_1823	4.1 down	47	No putative domains have been detected. A part of a putative operon with the downstream genes. TM

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13M	3.7	24.3	Outer membrane protein (OprG)	ABO_1922	H	-	A major outer membrane protein of <i>Ps. aeruginosa</i> (18); closest homology to <i>V.cholerae</i> ompW; is probably involved in low-affinity iron uptake (52).
57M	6.7	23.3	Membrane protein (putative)	ABO_1971	3.7 down	-	Predicted divalent heavy-metal cations transporter.
10M	7.0	86.1	Conserved hypothetical protein	ABO_2083	12.0 up	-	ANK, ankyrin repeats; ankyrin repeats mediate protein-protein interactions in very diverse families of proteins.
70M	5.2	39.8	Outer membrane phospholipase A precursor	ABO_2104	13 down	-	Outer membrane phospholipase A is an outer membrane-localized enzyme. It is implicated in the virulence of several pathogens.
17C	4.4	43.8	Conserved hypothetical protein	ABO_2153	4.6 down	-	No putative domains have been detected.
72M	6.4	58.5	Membrane protein (putative)	ABO_2547	18 down	-	Uncharacterized iron-regulated membrane protein. TM
60M	4.8	65.1	Inner membrane protein	ABO_2753	4.3 down	48	This family of proteins is required for the insertion of integral membrane proteins into cellular membranes. Can be associated with respiratory chain complexes.

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