# Analysis of storage lipid accumulation in *Alcanivorax borkumensis*: Evidence for alternative triacylglycerol biosynthesis routes in bacteria

**Running title:** Storage lipid biosynthesis in *Alcanivorax borkumensis*

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# ABSTRACT

Marine hydrocarbonoclastic bacteria like *Alcanivorax borkumensis* play a globally important role in bioremediation of petroleum oil contaminations in marine ecosystems. Accumulation of storage lipids, serving as endogenous carbon and energy sources during starvation periods, might be a potential adaptation mechanism to cope with nutrient limitation which is a frequent stress factor challenging those bacteria in their natural marine habitats. Here we report on the analysis of storage lipid biosynthesis in *A. borkumensis* strain SK2. Triacylglycerols (TAGs) and wax esters (WEs), but not poly(hydroxyalkanoic acids), are the principal storage lipids present in this and other hydrocarbonoclastic bacteria. Although so far assumed to be a characteristic restricted to Gram-positive actinomycetes, substantial accumulation of TAGs amounting up to a corresponding fatty acid content of more than 23% of the cellular dry weight is the first description of large-scale *de novo* TAG biosynthesis in a Gram-negative bacterium. The acyltransferase AtfA1 (ABO_2742) exhibiting wax ester synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT) activity plays a key role in both TAG and WE biosynthesis, whereas AtfA2 (ABO_1804) was dispensable for storage lipid formation. However, reduced but still substantial residual TAG levels in *atfA1* and *atfA2* knock-out mutants compellingly indicate the existence of a yet unknown WS/DGAT-independent alternative TAG biosynthesis route. Storage lipids of *A. borkumensis* were enriched in saturated fatty acids and were accumulated as insoluble intracytoplasmic inclusions exhibiting a huge structurally variety. Storage lipid accumulation provided only a slight growth advantage during short-term starvation periods but was not required for maintaining viability and long-term persistence during extended starvation phases.



# KEYWORDS

Acyl-CoA:diacylglycerol acyltransferase, *Alcanivorax*, hydrocarbonoclastic, persistence, starvation, storage lipid, triacylglycerol, wax ester, wax ester synthase.

# INTRODUCTION

Approximately 2-6 million tons of crude petroleum oil enter marine environments per annum mainly from anthropogenic sources, but also pollution from natural marine oil seepages results in a considerably input of petroleum oil into the sea. Petroleum oil is highly toxic to the majority of living organisms and can be tolerated only by relatively few species. Oil pollutions are therefore serious threats to the sensible marine ecosytems and result in severe ecological perturbations.

Fortunately, a substantial proportion of the petroleum oil entering marine habitats is degraded by indigenous microorganisms. Although many marine bacteria are capable of degrading petroleum hydrocarbons, only few of them seem to be important for petroleum biodegradation in natural marine environments. They belong to a new taxonomic group of phylogenetically related oil-degrading $\gamma$-proteobacteria which have been discovered during the last decade from different sites all over the world. These so-called hydrocarbonoclastic bacteria so far comprise the genera *Alcanivorax* (42), *Cycloclasticus* (10), *Marinobacter* (15), *Neptunomonas* (21), *Oleiphilus* (16), *Oleispira* (41) and *Thalassolituus* (40). Among those bacteria particularly members of the genus *Alcanivorax* seem to play a major role in the first steps of petroleum oil biodegradation. They are assumed to be of global importance for removal of crude oil contaminations in marine environments due to their cosmopolitan distribution (18, 19, 25).

*Alcanivorax* sp. and other hydrocarbonoclastic bacteria exhibit a unique oligotrophic physiology. They are specialized for hydrocarbon degradation but have an otherwise highly restricted substrate spectrum being capable of utilizing only a few organic acids like acetate and pyruvate, but not simple sugars, for growth (42). *Alcanivorax* spp. are present only in low abundance in pristine waters, but they multiply and grow rapidly in oil-polluted waters where they can constitute 80-90% of the microbial community (19, 25, 36). After this initial bloom and rapid increase in abundance, the population size declines to much lower numbers within a few weeks correlating with the biodegradation of the major portion of saturated hydrocarbons (20).

Despite the severe ecological problems caused by oil pollutions, the vast majority of the worlds´ oceans is still not polluted with petroleum oil. In fact, the marine habitat constitutes a quite nutrient limited environment, and oil spills are rather rare events of relatively small areal dissemination. In view of their oligotrophic lifestyle and the only sporadic availability of hydrocarbons as substrate for growth, it seems obvious that marine hydrocarbonoclastic bacteria are frequently facing extended phases of starvation conditions. Therefore, these bacteria must have developed strategies and abilities to adapt to and survive under such unfavourable environmental conditions which probably are important factors contributing to their successful ubiquitous distribution.

The accumulation of intracellular carbon storage compounds like storage lipids may be one of those strategies for survival of starvation periods. Storage lipids occur very frequently among hydrocarbon-utilizing marine bacteria with most of them accumulating specialized polymeric lipids like poly(hydroxybutyrate) or other poly(hydroxyalkanoic acids) (PHAs) (1). Beside PHAs, representing the most abundant class of lipophilic storage compounds produced by bacteria (33), less frequently also triacylglycerols (TAGs) and wax esters (WEs) are found as prokaryotic storage lipids. The accumulation of TAGs in large quantities has so far only been described for certain Gram-positive bacteria of the actinomycetes group belonging to the genera *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Streptomyces* (2). In contrast, WEs (oxoesters of long-chain primary fatty alcohols and long-chain fatty acids), along with minor amounts of TAGs, seem to be common storage lipids within the Gram-negative genus *Acinetobacter* (12, 13, 14, 22). Recently, the key enzyme for storage lipid biosynthesis in *Acinetobacter baylyi* strain ADP1 (formerly *Acinetobacter* sp. strain ADP1 (38)) was discovered, which is the wax ester synthase/acyl-coenzyme A:diacylglycerol acyltransferase (WS/DGAT). This unspecific acyltransferase simultaneously synthesizes WEs and TAGs by utilizing fatty acid coenzyme A thioesters (acyl-CoA) in addition to long-chain fatty alcohols or diacylglycerols (DGAs) as substrates, respectively. Remarkably, this novel enzyme exhibits no homology to known acyltransferases involved in

TAG or WE biosynthesis in eukaryotes, and it is also widely distributed among TAG-accumulating actinomycetes (23).

The recently finished genome sequencing project of *Alcanivorax borkumensis* (17, 31) prompted us to search for the presence of genes with potential involvement in storage lipid biosynthesis. The identification of two genes homologous to the WS/DGAT from *A. baylyi* strain ADP1 indicated the potential capability of *A. borkumensis* to synthesize and accumulate TAGs and/or WEs. This assumption was corroborated by the recently description of WE formation in the hydrocarbonoclastic strain *Alcanivorax jadensis* (formerly *Fundibacter jadensis*) (4). This present study now describes the analysis of biosynthesis and accumulation of storage lipids in *A. borkumensis* and addresses the role of the two WS/DGAT-homologous proteins in this pathway by biochemical characterization and directed mutagenesis.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** The strains and plasmids used in this study are listed in Table 1. *A. borkumensis* strains were cultivated aerobically at 30 °C in ONR7a medium containing 1% (wt/vol) sodium pyruvate or 0.5% (vol/vol) hexadecane for 72 h as described previously (42). *Marinobacter hydrocarbonoclasticus* (72 h at 30 °C), *Alcanivorax jadensis* T9 (72 h at 30 °C) and *Thalassolituus oleivorans* (96 h at room temperature) were grown aerobically in ONR7a medium containing 1% (wt/vol) sodium pyruvate. Cells of *E. coli* were grown in LB medium at 37 °C (30). Media were inoculated 1% (vol/vol) from saturated precultures. Solid media contained 1.8% (wt/vol) agar-agar. If appropriate, antibiotics were added at the following concentrations: ampicillin (Ap) 75 mg $l^{-1}$, kanamycin (Km) 50 mg $l^{-1}$, streptomycin (Sm) 100 mg $l^{-1}$, chloramphenicol (Cm) 34 mg $l^{-1}$, nalidixic acid (Ndx) 10.0 mg $l^{-1}$, tetracycline (Tc) 12.5 mg $l^{-1}$.

**Preparation of crude extracts.** Cells of *A. borkumensis* strains were cultivated with pyruvate as described above. Cells of *E. coli* were grown in LB medium to an $OD_{600\ nm}$ of 0.5 at 37 °C before IPTG was added to a final concentration of 1 mM, and induced cultures were incubated at 37 °C for 3 h. Cells were finally harvested by centrifugation at 4,000 × *g* for 20 min at 4 °C, washed twice with 125 mM sodium phosphate buffer (pH 7.4) and resuspended in the same buffer. Cell disruption was done by ultrasonification. Protein concentrations were determined by the Bradford method (3).

**Determination of enzyme activities.** WS/DGAT activity was determined in a total volume of 250 µl containing 12.5 µg $ml^{-1}$ bovine serum albumin (BSA), 4.72 µM [1-$^{14}$C]palmitoyl-CoA (specific activity 1.961 Bq $pmol^{-1}$; Hartmann Radiochemicals Braunschweig, Germany), 125 mM sodium phosphate buffer (pH 7.4) and different acceptor molecules at a concentration of 3.75 mM. 1-Hexadecanol and 1,2-dipalmitin were used as standard substrates for assaying WS and DGAT activity, respectively. Water insoluble substrates and BSA were applied as double concentrated stock solutions emulsified by ultrasonification. The assays were incubated for 20 min at 35 °C, and the reactions were stopped by extraction with 500 µl chloroform/methanol (1:1, vol/vol). After centrifugation, the chloroform phase was withdrawn, evaporated to dryness, and 40 µg of unlabeled reference substances were added. The reaction products were separated by TLC using different solvent systems for the separation of linear, cyclic and aromatic WEs, monoacylglycerols (MAGs), DAGs or TAGs, respectively, essentially as described previously (35). After separation of lipids by TLC and staining of TLC plates with iodine vapor, spots corresponding to the reaction products were scraped from the plates, and radioactivity was measured by scintillation counting. If reference substances were not available, the radioactive reaction products on the TLC plates were detected by autoradiography.

**DNA manipulations.** DNA manipulations and other standard molecular biology techniques were performed according to reference 30. The oligonucleotide primers used for PCR amplifications and RT-PCR analysis are listed in Table 2.

**Cloning of *atfA1* and *atfA2* from *A. borkumensis* SK2.** The *atfA1* and *atfA2* genes were amplified from total genomic DNA of *A. borkumensis* SK2 by tailored PCR using the oligonucleotides atfA1_5-end and atfA1_3-end or atfA2_5-end and atfA2_3-end (Table 2) as primers, respectively. The resulting PCR products were cloned as *Hin*dIII-*Xho*I fragments into the expression vector pET23a colinear to the T7-promoter yielding pET23a::*atfA1* or pET23a::*atfA2*, respectively, and were then transformed into *E. coli* BL21(DE3).

**Gene disruption by biparental filter mating.** For gene inactivation of *atfA1*, an ΩKm cassette was isolated by *Sma*I digestion of plasmid pSKsymΩKm and cloned into the singular *Stu*I site of *atfA1* yielding pET23a::*atfA1*ΩKm. For gene inactivation of *atfA2*, an ΩSm cassette was amplified by PCR from vector pCDFDuet-1 employing the primers $\Omega Sm^r$_5-end and $\Omega Sm^r$_3-end (Table 2), digested with *Sma*I and cloned into the singular *Ehe*I site of *atfA2* yielding pET23a::*atfA2*ΩSm. The plasmids were then linearized by *Hin*dIII digestion, fused to the *Hin*dIII restricted mobilizeable suicide plasmid pSUP202 yielding plasmids pSUP202::pET23a::*atfA1*ΩKm or pSUP202::pET23a::*atfA2*ΩSm, respectively, and were then transformed into *E. coli* S17-1.

Subsequently, gene insertion inactivation of the *atfA1* and *atfA2* gene in *A. borkumensis* was achieved by conjugational transfer of the suicide plasmids pSUP202::pET23a::*atfA1*ΩKm and pSUP202::pET23a::*atfA2*ΩSm, respectively, from *E. coli* strain S17-1 (donor) to *A. borkumensis* strains SK2 or *atfA1*ΩKm (recipients) employing a biparental filter mating technique. The donor strain was cultivated at 30 °C in LB medium for 24 h, the recipient strain was grown in ONR7a medium containing pyruvate for 48 h at 30 °C. Cells were harvested, washed once with cLB mating medium [LB medium supplemented with: 2% (wt/vol) pyruvate, 0.0445% (wt/vol) $Na_2HPO_4$ x $2H_2O$, 0.25% (wt/vol) $NaNO_3$, 1.15% (wt/vol) NaCl, 0.375% (wt/vol) KCl, 0.0735% (wt/vol) $CaCl_2$ x 2 $H_2O$] and concentrated 10-fold in cLB mating medium. Donor and recipient were mixed at a ratio of 1:4 (vol/vol), and 200 µl of this cell mixture were spotted on Millipore nitrocellulose membrane filters (45 mm diameter, 0.45 µm pore size) and placed onto cLB mating agar plates. After 24 h incubation at 30 °C cells were washed from the filters with 10 mM $MgSO_4$, and aliquots were plated onto

ONR7a agar plates supplemented with pyruvate containing appropriate antibiotics for selection of transconjugants (Km or Sm) and Ndx to select against *E. coli*. After incubation at 30 °C for 7 days, the resulting transconjugants were picked and patched onto selective agar plates with or without Cm. Putative homozygous gene disruptant mutants resulting from homologous recombination with a double crossover event were identified by their Cm sensitivity.

**Transcription analysis of *atfA1*, *atfA2*, *aceA* and Abo_2347 in *A. borkumensis* SK2.** RNA from cells cultivated with pyruvate was isolated using the RNeasy RNA purification kit (Qiagen, Hilden, Germany) according to the manufacturer´s protocol. RT-PCR was performed using the Qiagen OneStep RT-PCR-kit (Qiagen, Hilden, Germany) according to the provided instruction using the oligonucleotides atfA1_int1 and atfA1_int2 for analysis of *atfA1*, atfA2_int1 and atfA2_int2 for analysis of *atfA2*, aceA_int1 and aceA_int2 for analysis of *aceA*, or Abo_2743_int1 and Abo_2743_int2 for analysis of Abo_2743, respectively, as primers (see Table 2), and employing 0.5 ng RNA as template. Controls for DNA contaminations were done by adding the RNA template after the reverse transcriptase step before activating the *Taq*-polymerase.

**Thin-layer chromatography (TLC).** TLC analysis of lipid extracts from whole cells was done as described previously (23) using the solvent system hexane:diethylether:acetic acid (80:20:1, vol/vol/vol) for WE and TAG analysis. Lipids were visualized by staining with iodine vapor. Oleyl oleate and triolein were used as reference substances for WEs and TAGs, respectively.

**Fatty acid analysis.** Fatty acid analysis of whole cells was done by gas liquid chromatography (GC) according to reference (24) after derivatization to fatty acid methyl esters by sulfuric acid catalyzed methanolysis. Fatty acid methyl esters were analyzed by GC on an Agilent 6850 GC (Agilent Technologies, Waldbronn, Germany) equipped with a BP21 capillary column (50 m x 0.22 mm, film thickness 250 nm, SGE, Darmstadt, Germany) and a flame ionization detector (Agilent Technologies, Waldbronn, Germany). A 2 µl portion of the organic phase was analyzed after split injection (1:5); hydrogen (constant flow 0.6 ml $min^{-1}$)

was used as carrier gas. The temperatures of the injector and detector were 250 °C and 275 °C, respectively. The following temperature program was applied: 120 °C for 5 min, increase of 3 °C $min^{-1}$ to 180 °C, increase of 10 °C $min^{-1}$ to 220 °C, 220 °C for 31 min. Substances were identified by comparison of their retention times with those of authentic standard fatty acid methyl esters.

**Transmission electron microscopy (TEM).** After washing three times in PBS (pH 7.3), the cells were fixed with 2.5% (wt/vol) glutaraldehyde in 0.1 M PBS (pH 7.3) for 16 h. After three washing steps in PBS, each for 20 min, the cells were postfixed employing 1% (wt/vol) osmium tetroxide in 0.1 M PBS (pH 7.3) for 90 min and washed once with PBS for 15 min. Then samples were dehydrated by a graded water–ethanol series (30%, 50%, 70%, 90%, 96% and 100% ethanol and propylene oxide) each step for 15 min. For thin sectioning, the samples were embedded in SPURR resin with 50% (w/v) propylene oxide for 4 h and resin with 33% (w/v) propylene oxide for 16 h. The SPURR resin was changed every 24 h for a period of 3 days. The polymerization of the resin was performed at 70°C for 48 h. Sections with a thickness of 70–80 nm were made with an Ultracut Microtome (LEICA Mikroskopie und Systeme GmbH, Germany) using a diamond knife and were subsequently placed on a 200 mesh copper grid. Imaging was performed with a H-500 TEM (Hitachi, Japan) in the bright-field mode at 75 kV acceleration voltage at room temperature.

**Starvation experiments.** Cells of *A. borkumensis* strains were cultivated with pyruvate as described above to allow for accumulation of storage lipids. Cells were harvested, washed three times in ONR7a medium without any carbon source, resuspended in the same medium and incubated at 30 °C. At various time points aliquots of appropriate dilutions were plated onto ONR7a solid medium containing pyruvate for determination of viable cell counts.

# RESULTS

**Identification of two genes (*atfA1* + *atfA2*) coding for WS/DGAT-homologous proteins in *A. borkumensis*.** By using the WS/DGAT from *A. baylyi* strain ADP1, which is the first and best characterized member of this novel family of prokaryotic acyltransferases (23, 35), as template, two ORFs termed *atfA1* (ABO_2742) and *atfA2* (ABO_1804) were identified in the *A. borkumensis* SK2 genome sequence by BlastP search whose translational products revealed significant homologies. AtfA1 exhibited 49.1% and AtfA2 40.2% amino acid identity to WS/DGAT, respectively, whereas AtfA1 and AtfA2 shared 46.0% amino acid identity to each other. Both acyltransferase candidates comprised a conserved putative active site motif (HH*XXX*DG; see Fig. 1B) which has been proposed to be essential for catalytic activity (23).

Neither *atfA1* nor *atfA2* were clustered with genes with any known relevance for storage lipid biosynthesis (Fig. 1A). Interestingly, *atfA1* is situated in close proximity to the origin of replication of the chromosome probably resulting in a high gene dosage.

**Heterologous expression of *atfA1* and *atfA2* and biochemical characterization of the encoded acyltransferases.** The genes *atfA1* and *atfA2* were heterologously expressed in *E. coli* BL21(DE3) by employing the T7 promoter- and polymerase-based pET system. Although SDS-PAGE analysis revealed that most of the proteins were expressed as insoluble inclusion bodies (data not shown), functional expression of *atfA1* as well as *atfA2* in active form was achieved by using this system (Table 3). High levels of WS and a lower DGAT activity were detected for AtfA1. In contrast, AtfA2 exhibited only a substantial WS activity whereas DGAT activity was negligible (Table 3).

Both enzymes were then further characterized biochemically by analyzing the specificity of these acyltransferases for various acyl acceptor molecules. Both enzymes could comparably utilize a broad range of short, medium and long chain-length linear alcohols with highest specificity for medium chain-length alcohols (1-decanol) (Table 4). Furthermore, both acyltransferases were also highly active with cyclic or phenolic alcohols (Table 4). In clear

contrast to this, dramatic differences were observed between AtfA1 and AtfA2 regarding their specificity for different acylglycerol substrates (Table 5). Whereas AtfA2 highly efficiently utilized all tested MAGs, AtfA1 showed a clear preference for 1-MAG. Most strikingly, only AtfA1 possessed significant DGAT activity, whereas AtfA2 was virtually completely inactive with both DAGs used as substrate (Table 5). In summary, these results unequivocally demonstrated that AtfA1 and AtfA2 are truly potent acyltransferases, both exhibiting a very broad but substantially differing substrate specificity.

**TAGs and WEs are the principal storage lipids in *A. borkumensis*.** The *A. borkumensis* genome comprises two differentially expressed putative PHA synthase genes (*phaC1 + phaC2*) which is a hint for eventual presence of PHAs in this bacterium (29). The identification of two WS/DGAT-homologous acyltransferases now indicated that also TAGs and/or WEs might be synthesized in *A. borkumensis*. We therefore tested the presence of potential storage lipid species in alkane-grown and pyruvate-grown cells of *A. borkumensis* by means of GC and TLC analyses. Despite the existence of two PHA synthase genes, we were unable to detect significant amounts of any 3-hydroxyfatty acid, which are the monomer constituents of PHAs, by GC analysis regardless of the carbon source used for cultivation (in total less than 1% of the cellular dry weight, CDW). In conclusion, PHAs could be present only in irrelevant amounts in *A. borkumensis* under those conditions.

In contrast, large amounts of TAGs were accumulated in cells cultivated with pyruvate, whereas WEs were not produced under such conditions (Fig. 2B). However, WEs were synthesized together with lower amounts of TAGs when hexadecane was used as substrate. Beside TAGs and WEs, numerous other lipophilic substances of yet unknown chemical structure could be detected by TLC analysis. However, they were only of rather low abundance and were not further analyzed in this study (Fig. 2B). The neutral lipids mainly consisting of TAGs amounted up to a corresponding cellular fatty acid content of 23.2% of the CDW during growth on pyruvate, whereas the amounts of accumulated lipids were lower when the cells were cultivated with hexadecane (total fatty acid content 9.2% of CDW) (Table

6). Interestingly, TAGs isolated from pyruvate-grown cells exhibited a significant higher content of saturated fatty acids (particularly palmitic acid) compared to the total lipid fatty acid composition. The storage lipids produced during cultivation with hexadecane even almost exclusively contained saturated fatty acids although monounsaturated fatty acids (oleic acid + palmitoleic acid) still constituted a significant portion (33.8 mol-%) of the total cellular fatty acids under these culture conditions (Table 6). TAGs and WEs isolated from these cells contained beside palmitic acid (main constituent) and myristic acid (minor constituent) only 2.1 mol% or even only traces of unsaturated fatty acids, respectively (Table 6). This strong bias for saturated fatty acids indicates that the acyltransferases involved in TAG and WE biosynthesis in *A. borkumensis* strain SK2 probably have a high preference for saturated acyl-CoAs, although this aspect was not confirmed experimentally in this study. Hexadecanol, derived from oxidation of the growth substrate hexadecane, was the only fatty alcohol component of WEs isolated from alkane-grown cells detectable by GC analysis (data not shown).

**Functional analysis of *atfA1* and *atfA2* in *A. borkumensis* by directed gene insertion mutagenesis.** For evaluating the functional role of the acyltransferases AtfA1 and AtfA2 and their relative contribution to storage lipid biosynthesis in *A. borkumensis*, we generated isogenic *atfA1* and *atfA2* single knock-out mutants as well as a double knock-out mutant defective in both genes. This was achieved by disrupting the coding gene regions via insertion of a kanamycin or streptomycin gene cassette, respectively. Loss of acyltransferase activities in recombinant *E. coli* proved that by this both enzymes were efficiently inactivated (Table 3). Homozygous gene disruption mutants generated by homologous recombination via a double crossover event in *A. borkumensis* were obtained employing a conjugative biparental filter mating technique. Correct gene replacement in the mutant strains was confirmed by diagnostic PCR employing oligonucleotide primers binding to DNA regions up- and downstream of *atfA1* or *atfA2*, respectively, which were not present in the constructs used for gene inactivation (Fig. 2A).

Wild-type *A. borkumensis* SK2 cultivated with pyruvate exhibited a moderate level of WS activity of 64.0 pmol (mg min)$^{-1}$ and an about fourfold lower DGAT activity (Table 3). Inactivation of *atfA1* led to a drastic reduction (approximately 80%) of both WS as well as DGAT activity *in vitro* leaving only a very low residual level of DGAT activity left (2.5 pmol (mg min)$^{-1}$). In contrast, inactivation of *atfA2* caused only a relatively low reduction of both acyltransferase activities. In the double knock-out mutant WS activity was completely abolished, whereas a very low but significant basic level of DGAT activity was still maintained (Table 3).

In parallel to the highly diminished acyltransferase rates observed *in vitro*, inactivation of *atfA1* resulted in a strong decrease of TAG accumulation during cultivation on pyruvate or hexadecane as revealed by TLC analysis (Fig. 2B). Furthermore, during growth on hexadecane, biosynthesis of WEs was totally abrogated in the *atfA1* knock-out mutant. In utter contrast, disruption of *atfA2* did not seem to have any effect on TAG or WE accumulation under either culture condition as revealed by TLC. In addition, also no significant alterations in the profile of other neutral lipids were observed (Fig. 2B). Remarkably, even in the *atfA1*ΩKm*atfA2*ΩSm double knock-out mutant reduced but still substantial amounts of TAGs were present in cells cultivated on either substrate (Fig. 2B) accounting for a fatty acid content of ca. 5-10% of the CDW (Table 6). Strains with inactivated *atfA1* gene (*atfA1*ΩKm and *atfA1*ΩKm*atfA2*ΩSm) exhibited an altered fatty acid composition comprising a lower palmitic acid and a higher unsaturated fatty acid content, whereas disruption of *atfA2* had no effect on the fatty acid profile (Table 6).

In order to rule out that the phenotypes observed in *atfA1* knock-out strains were influenced by polar effects, we performed RT-PCR analyses of the adjacent genes *aceA* and Abo_2743 (Fig. 1A). Both genes were similarly expressed in the wild type and in the *atfA1*ΩKm mutant during cultivation with pyruvate confirming that polar effects on neighbouring genes caused by *atfA1* disruption can be excluded (data not shown).

Although showing distinct acyltransferase activity *in vitro*, loss of AtfA2 function had surprisingly no detectable influence on storage lipid accumulation *in vivo*, suggesting that

*atfA2* might be silent in *A. borkumensis*. However, RT-PCR analysis demonstrated that *atfA2* was definitely expressed in the cells (Fig. 1C). Functional expression of *atfA2* was also documented by the fact that somehow reduced WS activity levels were observed *in vitro* in *atfA2* knock-out mutants (Table 3).

**Ultrastructure of intracellular storage lipid inclusions in *A. borkumensis*.** Storage lipids predominantly consisting of TAGs synthesized during cultivation of *A. borkumensis* on pyruvate were accumulated in form of electron-transparent intracytoplasmic inclusions as shown by TEM (Fig. 3A-D). Remarkably, these lipid inclusions were highly inhomogeneous in size and shape, mainly comprising spherical inclusions, but also rectangular, disc-shape, needle-like and irregularly shaped inclusions occurred (Fig. 3A-D). During growth on hexadecane, intracellular storage lipids consisted of TAGs and WEs (Fig. 2B). Under these conditions spherical inclusions were only very rarely present (Fig. 3G-J). The structures of the inclusions were generally highly variable, many of them being quite long and sprawled, occasionally reaching a length of 500 nm. Albeit the double knock-out mutant was still capable to accumulate significant amounts of TAGs (Fig. 2B), TEMs of the *A. borkumensis* strain *atfA1*ΩKm*atfA2*ΩSm cells did not show obvious lipid inclusions, neither in pyruvate-grown (Fig. 3E+F) nor hexadecane-grown cells (Fig. 3K+L).

**Role of storage lipid accumulation for survival of carbon starvation.** Cells of *A. borkumensis* wild type and of the double knock-out mutant were preincubated under conditions promoting storage lipid accumulation and were then transferred to carbon source free medium. Survival of the cells under these conditions was then monitored by measuring remaining viable cell counts (colony forming units, CFUs) over the time course of carbon starvation (Fig. 2C). Wild type cells showed a two log-fold decrease in viability within the first 10 days of starvation, but after that viability remained relatively constant even over an extended period of starvation of 4 weeks. Storage lipid-reduced cells of the double knock-out mutant seemed to lose viability slightly more rapidly between days 2-8 of starvation

compared to the wild type, but finally also here only a two log-fold decrease was observed also reaching a plateau after 10 days after which no further decrease in viability occurred even after a prolonged starvation period (Fig. 2C).

**Occurrence of storage lipids in other hydrocarbonoclastic bacteria.** As already described earlier, *A. borkumensis* accumulates only TAGs during cultivation with pyruvate, but no WEs are synthesized *de novo* (Fig. 2B). We then investigated other hydrocarbonoclastic bacteria for their ability to accumulate neutral storage lipids under these conditions (Fig. 4). In contrast to *A. borkumensis*, all three investigated hydrocarbonoclastic strains were able to synthesize substantial amounts of WEs *de novo*. In addition to this, only *Alcanivorax jadensis* produced also substantial amounts of TAGs simultaneously, whereas *Marinobacter hydrocarbonoclasticus* accumulated only low levels and *Thalassolituus oleivorans* just trace amounts of TAGs (Fig. 4). In all tested strains, no significant amounts of 3-hydroxyfatty acid monomers were detectable by GC analysis indicating that PHAs were absent or present at only very low amounts (data not shown).

## DISCUSSION

In this study we have demonstrated the ability of the hydrocarbonoclastic marine bacterium *A. borkumensis* to synthesize and to intracellularly accumulate large amounts of TAGs *de novo* from the unrelated substrate pyruvate amounting up to a corresponding fatty acid content of more than 23% of the CDW. In Gram-negative bacteria, TAGs have been reported so far only as a minor constituent in *Acinetobacter* species (23, 28). Thus, to the best of our knowledge this is the first description of substantial TAG accumulation in a Gram-negative prokaryote. Furthermore, we refute hereby the so far largely accepted assumption that occurrence of large scale TAG accumulation in bacteria is restricted to the Gram-positive group of actinomycetes (2, 39).

Despite the presence of two differential expressed PHA synthase genes in *A. borkumensis* SK2 (29), we showed that only TAGs and WEs are the predominating hydrophobic storage compounds occurring in this strain. Although very low amounts of PHAs might be synthesized by *A. borkumensis*, our data prove that PHAs do clearly play no role as storage compound under the tested cultivation conditions. This is corroborated by the fact that no obvious intracellular lipophilic inclusions remain in mutant cells impaired in TAG and WE biosynthesis. PHA accumulation has been occasionally described for some other hydrocarbonoclastic bacteria (5, 11), but this was solely based on positive staining results employing lipophilic dyes and was not supported by chemical analyses. Such lipophilic dyes are not specific and can not differentiate between PHAs, TAGs, WEs and other neutral lipids like hydrocarbon inclusions. Based on our results in *A. borkumensis* and some other selected species it can be assumed that most probably TAGs and WEs, but not PHAs, are the principal storage lipids generally present in hydrocarbonoclastic marine bacteria. Since genes coding for key enzymes such as glycogen synthase, ADP-glucose pyrophosphorylase or cyanophycin synthetase required for biosynthesis of other bacterial carbon storage compounds like glycogen or cyanophycin are lacking in the *A. borkumensis* genome (31), TAGs and WEs are most likely the only relevant carbon storage compounds produced in this bacterium.

The presence of two WS/DGAT-homologues in *A. borkumensis* SK2 actually suggests a redundant function. However, although both enzymes (AtfA1 + AtfA2) exhibited robust acyltransferase activity in *in vitro* tests, only AtfA1 seems to be involved *in vivo* in TAG and WE biosynthesis and in fact plays undoubtedly a pivotal role in storage lipid accumulation in this organism. Surprisingly, AtfA2 is fully dispensable for this process despite of contributing to the WS activity observed *in vitro* in *A. borkumensis*. Thus, due to the lack of an obvious phenotype, the function of this acyltransferase remains unclear, but it might be involved in production of a yet unknown fatty acid ester substance which could not be detected by the applied methods. Both acyltransferases exhibited little and partially overlapping substrate specificity; however, there were drastic differences regarding their

specificity towards acylglycerols as substrates. Specifically, AtfA2 was almost totally devoid of DGAT activity. Thus by sequence comparison of AtfA1 and AtfA2 from *A. borkumensis* and WS/DGAT from *A. baylyi*, amino acid residues conserved in AtfA1 and WS/DGAT, but not AtfA2, might specify those domains that are important for DGAT activity, whereas amino acids conserved among all three enzymes indicate regions that are probably necessary for general acyltransferase catalytic activity (Fig. 1B).

Remarkably, even in the double knock-out mutant a reduced but still substantial level of TAG accumulation remained although both WS/DGAT-homologues were inactivated. This is compelling evidence for the existence of an alternative, non WS/DGAT-dependent TAG-biosynthesis pathway in this bacterium. Trace amounts of residual TAGs in a WS/DGAT-knock-out mutant of *A. baylyi* has already earlier provided hints that such an alternative pathway may exist (23). Due to the much higher TAG content in *A. borkumensis* this now became here more obvious and unambiguous. In eukaryotes, three different routes for TAG formation are known all using DAG as substrate. Members of the eukaryotic DGAT1 and 2 protein families, which exhibit no sequence homologies to each other, catalyze the acylation of DAG using acyl-CoA as substrate in a similar manner like the bacterial WS/DGAT (6, 7). Beside this most widespread pathway, an acyl-CoA independent reaction for TAG synthesis is catalyzed by a phospholipid:DAG acyltransferase. This enzyme uses phospholipids as acyl donor for DAG esterification and has been found in plants and yeast (9). The third mechanism of TAG synthesis is described for DAG:DAG transacylases, which were identified in animals and plants utilizing DAG as acyl donor as well as acyl acceptor (26, 34). However, no genes coding for such DAG:DAG transacylases are known yet. The nature of the alternative TAG biosynthesis route in *A. borkumensis* is not known so far. The genome comprises numerous putative acyltransferase genes of yet unknown function (31; see Table S1 in the supplemental material), but none of these exhibits reasonable homology to any of the above mentioned eukaryotic TAG-synthesizing enzymes. Residual but fairly low DGAT activities still observable in *atfA1* knock-out strains of *A. borkumensis* make it questionable that an alternative, so far unknown DGAT isoenzyme is responsible for TAG biosynthesis in

these mutants. However, it has to be emphasized that such a potential DGAT isoenzyme might notwithstanding be present but only poorly active under the applied *in vitro* assay conditions due to differing requirements (pH, buffer, salts). Whether phospholipid:DAG acyltransferase or DAG:DAG transacylase activities are measurable in *A. borkumensis*, has not been examined in this study.

In contrast to the other three investigated hydrocarbonoclastic bacteria, *A. borkumensis* was only capable to synthesize WEs during cultivation on hexadecane, but not from the unrelated substrate pyruvate indicating that this strain is probably impaired in *de novo* formation of fatty alcohols. In *A. baylyi*, fatty alcohols are formed starting from long-chain acyl-CoAs by two consecutive NADPH-dependent reduction reactions catalyzed by a fatty acyl-CoA reductase and a yet unidentified fatty aldehyde reductase (28). The *A. borkumensis* genome does not comprise a fatty acyl-CoA reductase homologue (data not shown) which might be the probable reason for the lacking capability of *de novo* fatty alcohol biosynthesis in this bacterium. During oxidative degradation of alkanes, fatty alcohols are formed as first intermediate and are thus as such also available as substrate for AtfA1 enabling WE formation despite the absence of *de novo* fatty alcohol biosynthesis.

TAG and WE inclusions in lipid accumulating bacteria are usually organized in form of spherical intracytoplasmic lipophilic inclusions (overview in reference 39). In contrast to this, storage lipid inclusions in *A. borkumensis* showed an extremely high structural diversity comprising spherical, rectangular, disc-shape, needle-like and irregularly shaped forms. A peculiarity of storage lipids from *A. borkumensis*, compared to those from other bacteria, is their relatively low content of unsaturated fatty acids. In particular, WEs and TAGs produced during cultivation on hexadecane almost exclusively contained saturated fatty acids. Such unsaturated lipids are supposed to be largely solid at physiological temperatures, thus resulting in a relatively high degree of crystallinity, which in turn might then lead to the formation of such divers and inhomogeneous inclusion structures. Furthermore, this biased fatty acid composition also strongly indicates that AtfA1, the key enzyme for neutral lipid biosynthesis in strain SK2, probably has a high preference for saturated acyl-CoAs. Although

experimental proof for this is pending, the relative changes in the fatty acid profile caused by *atfA1* disruption corroborate that assumption.

Nutrient starvation is assumed to be an environmental stress factor frequently challenging marine hydrocarbonoclastic bacteria in their natural habitat. Accumulation of storage lipids serving as endogenous carbon source during starvation periods might thus be one conceivable strategy to cope with this and to prolong viability under such unfavourable conditions. Although we could not definitely address this hypothesis due to the lack of a completely storage lipid negative mutant, storage lipid accumulation seems to provide a growth advantage only during short periods of starvation but is not required for maintaining viability during long-term starvation phases. Interestingly, after the majority of the population (ca. 99%) rapidly lost viability already after a few days during starvation, a subpopulation of *A. borkumensis* went into a stable, non-replicating, but viable persistence state which allowed survival even during extended starvation periods. This long-term persistence capability, putatively involving the stringent response (8), might be a key factor for the successful global distribution of *A. borkumensis* and other hydrocarbonoclastic bacteria and for their adaptability to an ever changing environment.

## FOOTNOTES

[1] The abbreviations used are: CDW, cellular dry weight; CFU, colony forming unit; DGAT, acyl-CoA:diacylglycerol acyltransferase; DAG, diacylglycerol; MAG, monoacylglycerol; PHA, poly(hydroxyalkanoic acid); TAG, triacylglycerol; WE, wax ester; WS, wax ester synthase.

## ACKNOWLEDGMENTS

The study was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG) to A. S. (Ste 386/7-3). P. N. G. and K. N. T. acknowledge support from the German Ministry for Education and Research (BMBF) in the frame of the GenoMik network "Genome research on Bacteria Relevant for Agriculture, Environment and Biotechnology". The authors would like to thank Biggy-Nadine Wendt and Chlud Kaddor for skilful technical assistance.

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# FIGURE LEGENDS

**Fig. 1: Characterization of the WS/DGAT homologues AtfA1 and AtfA2 from *A. borkumensis* SK2. A)** Organization of the *atfA1* and *atfA2* gene loci. ABO_2740, hypothetical protein; *aceA*, isocitrate lyase; ABO_2743, hypothetical protein; *cspG*, cold-shock domain family protein; ABO_2745, hypothetical protein; ABO_1806, AMP-binding family protein; ABO_1805, hypothetical protein; ABO_1803, hypothetical protein; ABO_1802, CinA domain protein; *recA*, RecA protein. **B)** Multiple amino acid sequence alignment of the WS/DGAT enzyme AtfA from *A. baylyi* strain ADP1 and AtfA1 and AtfA2 from *A. borkumensis* SK2. Analysis was done using the CLUSTAL W program (37). Residues identical in all sequences are shaded in light gray, those only conserved in AtfA and AtfA1 are shaded in dark gray. A putative active site motif is boxed. **C)** Transcription analysis of *atfA1* and *atfA2*. Expression of *atfA1* and *atfA2* was analyzed by RT-PCR in samples derived from cells cultivated with 1% (wt/vol) sodium pyruvate as carbon source. St, *Pst*I-digested λ DNA. +, RT-PCR assay; –, control for DNA contaminations.

**Fig. 2: Characterization of *A. borkumensis atfA1* and *atfA2* gene disruption mutants. A)** Genotypic characterization of *A. borkumensis* mutants. Mutants were analyzed by diagnostic PCR using oligonucleotide primer pairs binding up- and downstream of the *atfA1* (1) or *atfA2* gene (2), respectively. Expected sizes: native *atfA1* (1.5 kbp), native *atfA2* (1.6 kbp), *atfA1*ΩKm (2.5 kbp), *atfA2*ΩSm (2.6 kbp). St, *Pst*I-digested λ DNA. **B)** TLC analyses of storage lipid accumulation in *A. borkumensis* mutants. Cells were cultivated in ONR7a medium containing 1% (wt/vol) sodium pyruvate or 0.5% (vol/vol) hexadecane, respectively, for 72 h and analyzed by TLC. Lipid extracts obtained from 1.5 mg lyophilized cells were applied per lane. A, oleic acid; B, triolein; C, oleyl oleate. 1, SK2; 2, *atfA1*ΩKm; 3, *atfA2*ΩSm; 4, *atfA1*ΩKm*atfA2*ΩSm. **C)** Survival of *A. borkumensis* strains SK2 (triangles) and *atfA1*ΩKm*atfA2*ΩSm (squares) during carbon starvation. After 72 h preincubation in ONR7a medium with pyruvate to allow for the accumulation of storage lipids, cells were transferred into ONR7a medium containing no carbon source, and survival was monitored by

determining viable cell counts. Values are means of duplicates ± error bars. Abbreviations: WE, wax esters; TAG, triacylglycerols; FA, free fatty acids; DAG, diacylglycerols; MAG, monoacylglycerols.

**Fig. 3: Structure of neutral lipid inclusions in *A. borkumensis*.** Cells were cultivated for 72 h in ONR7a medium containing 1% (wt/vol) sodium pyruvate (A-F) or 0.5% (vol/vol) hexadecane (G-L). Ultrathin sections of cells were analyzed by transmission electron microscopy. The scale bars correspond to 200 nm. *A. borkumensis* SK2 (A-D and G-J), *A. borkumensis atfA1*ΩKm*atfA2*ΩSm (E, F, K, L).

**Fig. 4: Storage lipid accumulation in different hydrocarbonoclastic marine bacteria.** Cells were cultivated in ONR7a medium containing 1% (wt/vol) sodium pyruvate for 72 h (*Marinobacter hydrocarbonoclasticus* and *Alcanivorax jadensis* T9) or 96 h (*Thalassolituus oleivorans*) and analyzed by TLC. Lipid extracts obtained from 1.5 mg lyophilized cells were applied per lane. 1, *M. hydrocarbonoclasticus*; 2, *T. oleivorans*; 3, *A. jadensis* T9. Abbreviations: see legend to Fig. 2.

**Table 1: Strains and plasmids used in this study.**

| Strain or plasmid | Relevant properties | Source or reference |
|---|---|---|
| *Alcanivorax borkumensis* | | |
| SK2 | Type strain, wild type | 42; DSM 11573 |
| *atfA1*ΩKm | *atfA1* disruption mutant; $Km^r$; derivative of SK2 | This study |
| *atfA2*ΩSm | *atfA2* disruption mutant; $Sm^r$; derivative of SK2 | This study |
| *atfA1*Ω*Km atfA2*Ω*Sm* | *atfA1* + *atfA2* double disruption mutant; $Km^r$; $Sm^r$; derivative of *atfA1*ΩKm | This study |
| *Alcanivorax jadensis* T9 | Type strain, wild type | 5, 11; DSM 12178 |
| *Marinobacter hydrocarbonoclasticus* | Type strain, wild type | 15; DSM 8798 |
| *Thalassolituus oleivorans* | Type strain, wild type | 40; DSM 14913 |
| *Escherichia coli* | | |
| BL21(DE3) | $F^-$, *ompT*, *hsdS*$_B$ ($r_B^-$, $m_B^-$), *gal*, *dcm* (DE3) | Novagen, Madison, USA |
| S17-1 | *recA*1, *thi*1, *hsdR*17($r_k$-, $m_k$+), *proA, tra*-RP4 | 32 |
| Plasmids | | |
| pET23a(+) | $Ap^r$; T7 promoter-based expression vector | Novagen, Madison, USA |
| pET23a::*atfA1* | Derivative of pET23a containing the *atfA1* gene as a 1.4-kbp *Hin*dIII/*Xho*I fragment; $Ap^r$ | This study |
| pET23a::*atfA2* | Derivative of pET23a containing the *atfA2* gene as a 1.4-kbp *Hin*dIII/*Xho*I fragment; $Ap^r$ | This study |
| pET23a::*atfA1*ΩKm | Ω$Km^r$ cassette cloned into *Stu*I site of pET23a::*atfA1*; $Ap^r$; $Km^r$ | This study |
| pET23a::*atfA2*ΩSm | Ω$Sm^r$ cassette cloned into *Ehe*I site of pET23a::*atfA2*; $Ap^r$; $Sm^r$ | This study |
| pSKsymΩKm | $Ap^r$; $Km^r$; contains an Ω$Km^r$ cassette | 27 |
| pCDFDuet-1 | $Sm^r$; source for Ω$Sm^r$ cassette construction | Novagen, Madison, USA |
| pSUP202 | $Ap^r$; $Cm^r$; $Tc^r$; ColE1 origin; mob site; unable to replicate in *A. borkumensis* | 32 |
| pSUP202:: pET23a::*atfA1*ΩKm | Fusion of pSUP202 and pET23a::*atfA1*ΩKm via *Hin*dIII sites; mob site; $Ap^r$; $Cm^r$; $Km^r$ | This study |
| pSUP202:: pET23a::*atfA2*ΩSm | Fusion of pSUP202 and pET23a::*atfA2*ΩSm via *Hin*dIII sites; mob site; $Ap^r$; $Cm^r$; $Sm^r$ | This study |

**Table 2: Oligonucleotides used in this study as primers for PCR, diagnostic PCR and RT-PCR.** Restriction sites used for cloning purposes are underlined. Start and stop codons of *atfA1* and *atfA2* are indicated in bold.

| Primer | Sequence |
|---|---|
| atfA1_5-end | 5´-TTTTTTAAGCTTAAGGAGAATAT**ATG**AAAGCGCTT-3´ |
| atfA1_3-end | 5´-TTTTTTCTCGAG**CTA**TTTAATTCCTGCACCGATTT-3´ |
| atfA2_5-end | 5´-TTTTTTAAGCTTAAGGAGCAGCAAGT**ATG**GCCCGT-3´ |
| atfA2_3-end | 5´-TTTTTTCTCGAG**TCA**AGGCTCCACCAGCG-3´ |
| atfA1_up | 5´-CAGCTGGCATGGAGAGTGCATAAC-3´ |
| atfA1_down | 5´-GATGCGGTTTAGTTCAGTTGCCAT-3´ |
| atfA2_up | 5´- GGCTGACTGTTGATAACCAAACGC-3´ |
| atfA2_down | 5´-CCCGTTAGCATGCCGGCAATGTG-3´ |
| atfA1_int1 | 5´-GACAGCAACCCATGCACGTAG-3´ |
| atfA1_int2 | 5´-GGTGAGGGCGGTGAAATTGAG-3´ |
| atfA2_int1 | 5´- AGCATCTGCCGCCCATTTAAC-3´ |
| atfA2_int2 | 5´-ACCGATCACGCCAAACTCAAG-3´ |
| aceA_int1 | 5´-GCGACGAAGCCAAGCAGAAAG-3´ |
| aceA_int2 | 5´-TCACAGTAGCGATGCCCTGAC 3´ |
| Abo_2743_int1 | 5´-GTGTTCAGCGGCAATATCAGC-3´ |
| Abo_2743_int2 | 5´-TGGAGCCAAAGTGAGCACATC-3´ |
| ΩSm$^r$_5-end | 5´-TTTTTTCCCGGGCTCACGCCCGGAGCGTAGCGACC-3´ |
| ΩSm$^r$_3-end | 5´-TTTTTTCCCGGGAACGACCCTGCCCTGAACCGACG-3´ |

**Table 3: WS and DGAT activities in *A. borkumensis* and *E. coli* strains.** Assays were done employing crude extracts corresponding to 50 µg total protein for recombinant *E. coli* strains or 100 µg total protein for pyruvate-grown *A. borkumensis* strains. Data are mean values of experiments done in triplicates ±SD. Control experiments without addition of acyl acceptor substrates revealed WS activities of 14.5 and 6.4 pmol (mg min)$^{-1}$ and DGAT activities of 10.3 and 0.3 pmol (mg min)$^{-1}$ for *E. coli* strains expressing *atfA1* or *atfA2*, respectively. 1 pmol (mg min)$^{-1}$ corresponds to 117.66 and 235.32 counts per minute for *E. coli* or *A. borkumensis* strains, respectively.

| **Strain** | **Specific enzyme activities** [pmol (mg min)$^{-1}$] | |
|---|---|---|
| | **WS** | **DGAT** |
| ***E. coli* BL21(DE3)** | | |
| pET23a | 0.4±0.1 | 0.2±0.0 |
| pET23a::*atfA1* | 308.2±37.9 | 131.7±4.3 |
| pET23a::*atfA2* | 237.0±30.5 | 4.6±0.8 |
| pET23a::*atfA1*ΩKm | 1.0±0.5 | 0.3±0.1 |
| pET23a::*atfA2*ΩSm | 4.9±0.3 | 0.3±0.1 |
| ***A. borkumensis*** | | |
| SK2 | 64.0±8.1 | 17.2±0.3 |
| *atfA1*ΩKm | 11.3±1.4 | 2.5±0.2 |
| *atfA2*ΩSm | 38.5±1.2 | 10.1±1.6 |
| *atfA1*ΩKm*atfA2*ΩSm | 0.4±0.2 | 1.6±0.8 |

**Table 4: Acyl-acceptor specificites of AtfA1 and AtfA2 from *A. borkumensis* SK2 for different alcohol substrates.** Assays were done employing crude extracts corresponding to 50 µg total protein from recombinant *E. coli* BL21(DE3) strains harbouring plasmid pET23a::*atfA1* or pET23a::*atfA2* for heterologous expression of AtfA1 or AtfA2, respectively. Values are indicated as relative specific activities in comparison to hexadecanol as acyl acceptor. Data are mean values of experiments done in triplicate ±SD. 100% activity are equivalent to 324.9 and 237.0 pmol (mg min)$^{-1}$ for AtfA1 and AtfA2, respectively.

| **Acyl acceptor** | **Activity** [% of hexadecanol control] | |
|---|---|---|
| | **AtfA1** | **AtfA2** |
| no acceptor | 4.4±1.2 | 0.3±0.0 |
| 1-Hexadecanol | 100.0±3.2 | 100.0±12.9 |
| 1-Butanol | 114.9±16.7 | 72.7±18.1 |
| 1-Decanol | 161.6±26.4 | 115.6±8.3 |
| 1-Tetracosanol | 18.2±4.2 | 7.5±2.2 |
| Cyclohexanol | 99.0±2.2 | 129.9±55.4 |
| Cyclohexylethanol | 131.6±9.4 | 167.0±13.4 |
| 2-Phenylethanol | 109.8±10.7 | 154.3±19.9 |

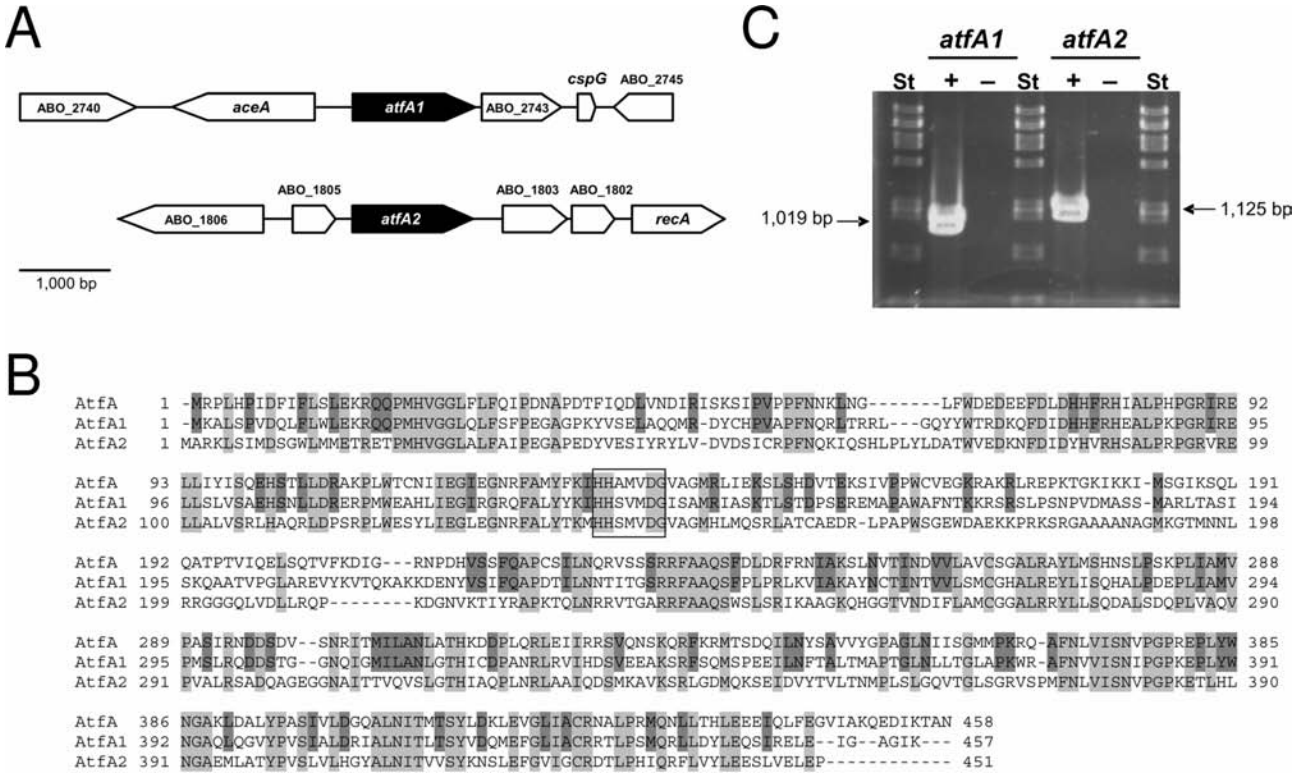
**Table 5: Acyl acceptor specificities of AtfA1 and AtfA2 from *A. borkumensis* SK2 for glycerol and acylglycerols.** Assays were done employing crude extracts corresponding to 50 µg total protein from recombinant *E. coli* BL21(DE3) strains harbouring plasmid pET23a::*atfA1* or pET23a::*atfA2* for heterologous expression of AtfA1 or AtfA2, respectively. 1,2- and 2,3-dipalmitoylglycerol as well as 1- and 3-monopalmitoylglycerol could not be separated under the applied TLC conditions and are therefore summed up. Data are averages of experiments done in triplicates ±SD. MPG, monopalmitoylglycerol; DPG, dipalmitoylglycerol; TPG, tripalmitoylglycerol; n.d., not detectable; n.t., not tested.

| **Acyl acceptor** | **Formation of** [pmol (mg min)$^{-1}$] | | | | |
|---|---|---|---|---|---|
| | **1- + 3-MPG** | **2-MPG** | **1,2- + 2,3-DPG** | **1,3-DPG** | **TPG** |
| **AtfA1** | | | | | |
| Glycerol | n.d. | n.d. | n.t. | n.t. | n.t. |
| 1-Monopalmitoylglycerol | | | 32±2 | 237±16 | n.t. |
| 2-Monopalmitoylglycerol | | | 85±27 | 41±10 | n.t. |
| 3-Monopalmitoylglycerol | | | 31±2 | 74±3 | n.t. |
| 1,2-Dipalmitoylglycerol | | | | | 181±9 |
| 1,3-Dipalmitoylglycerol | | | | | 100±36 |
| **AtfA2** | | | | | |
| Glycerol | n.d. | n.d. | n.t. | n.t. | n.t. |
| 1-Monopalmitoylglycerol | | | 22±1 | 345±27 | n.t. |
| 2-Monopalmitoylglycerol | | | 232±15 | 134±5 | n.t. |
| 3-Monopalmitoylglycerol | | | 19±1 | 378±33 | n.t. |
| 1,2-Dipalmitoylglycerol | | | | | 4±1 |
| 1,3-Dipalmitoylglycerol | | | | | 2±0 |

**Table 6: Fatty acid content and fatty acid composition of *A. borkumensis* strains and fatty acid composition of purified lipids isolated from strain SK2.** Cells were cultivated for 72 h in ONR7a medium with 1% (wt/vol) sodium pyruvate or 0.5% (vol/vol) hexadecane. Lyophilized cells were analyzed by GC. TAGs and WEs were purified from cells of the wild type (SK2) by preparative TLC prior subjection to GC analysis. n.d., not determined; tr, traces (<0.5mol-%).

| **Carbon source** | **Strain or lipid** | **Total fatty acids** | **Fatty acid composition** (mol-%) | | | | |
|---|---|---|---|---|---|---|---|
| | | (% of CDW) | **C14:0** | **C16:0** | **C16:1** | **C18:0** | **C18:1** |
| **Pyruvate** | SK2 | 23.2 | 5.1 | 46.8 | 9.7 | 3.3 | 35.1 |
| | *atfA1*ΩKm | 10.1 | 3.4 | 39.7 | 12.1 | 3.7 | 41.1 |
| | *atfA2*ΩSm | 26.9 | 5.1 | 47.1 | 9.4 | 3.4 | 35.0 |
| | *atfA1*ΩKm*atfA2*ΩSm | 9.6 | 2.9 | 36.8 | 13.9 | 5.2 | 41.2 |
| | TAGs purified from SK2 | n.d. | 7.0 | 60.8 | 5.0 | 4.4 | 22.7 |
| **Hexadecane** | SK2 | 9.2 | 8.5 | 55.3 | 11.5 | 2.4 | 22.3 |
| | *atfA1*ΩKm | 4.0 | 4.9 | 42.1 | 19.7 | 2.9 | 30.4 |
| | *atfA2*ΩSm | 11.7 | 9.5 | 54.5 | 10.8 | 2.6 | 22.7 |
| | *atfA1*ΩKm*atfA2*ΩSm | 5.3 | 5.3 | 42.5 | 19.4 | 2.8 | 30.1 |
| | TAGs purified from SK2 | n.d. | 15.8 | 76.0 | 1.4 | 5.9 | 0.7 |
| | WEs purified from SK2 | n.d. | 6.4 | 85.3 | tr | 8.3 | Tr |

1 FIG. 1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atfA1
atfA2
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AtfA1 1 -MKALSFVDQLFLWLEKRQQPMHVGGLQLFSFPEGAGPKYVSELAQQMR-DYCHPVAPFNQRLTRRL---GQYYWTRDKQFDIDHHFRHEALPKPGRIRE 95
AtfA2 1 MARKLSIMDSGWLMMETRETPMHVGGLALFAIPEGAPEDYVESIYRYLV-DVDSICRPFNQKIQSHLPLYLDATWVEDKNFDIDYHVRHSALPRPGRVRE 99
AtfA 93 LLIYISQEHSTLLDRAKPLWTCNIIEGIEGNRFAMYFKIHHAMVDGVAGMRLIEKSLSHDVTEKSIVPPWCVEGKRAKRLREPKTGKIKKI-MSGIKSQL 191
AtfA1 96 LLSLVSAEHSNLLDRERPMWEAHLIEGIRGRQFALYYKIHHSVMDGISAMRIASKTLSTDPSEREMAPAWAFNTKKRSRSLPSNPVDMASS-MARLTASI 194
AtfA2 100 LLALVSRLHAQRLDPSRPLWESYLIEGLEGNRFALYTKMHHSMVDGVAGMHLMQSRLATCAEDR-LPAPWSGEWDAEKKPRKSRGAAAANAGMKGTMNNL 198
AtfA 192 QATPTVIQELSQTVFKDIG---RNPDHVSSFQAPCSILNQRVSSSRRFAAQSFDLDRFRNIAKSLNVTINDVVLAVCSGALRAYLMSHNSLPSKPLIAMV 288
AtfA1 195 SKQAATVPGLAREVYKVTQKAKKDENYVSIFQAPDTILNNTITGSRRFAAQSFPLPRLKVIAKAYNCTINTVVLSMCGHALREYLISQHALPDEPLIAMV 294
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AtfA1 295 PMSLRQDDSTG--GNQIGMILANLGTHICDPANRLRVIHDSVEEAKSRFSQMSPEEILNFTALTMAPTGLNLLTGLAPKWR-AFNVVISNIPGPKEPLYW 391
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AtfA 386 NGAKLDALYPASIVLDGQALNITMTSYLDKLEVGLIACRNALPRMQNLLTHLEEEIQLFEGVIAKQEDIKTAN 458
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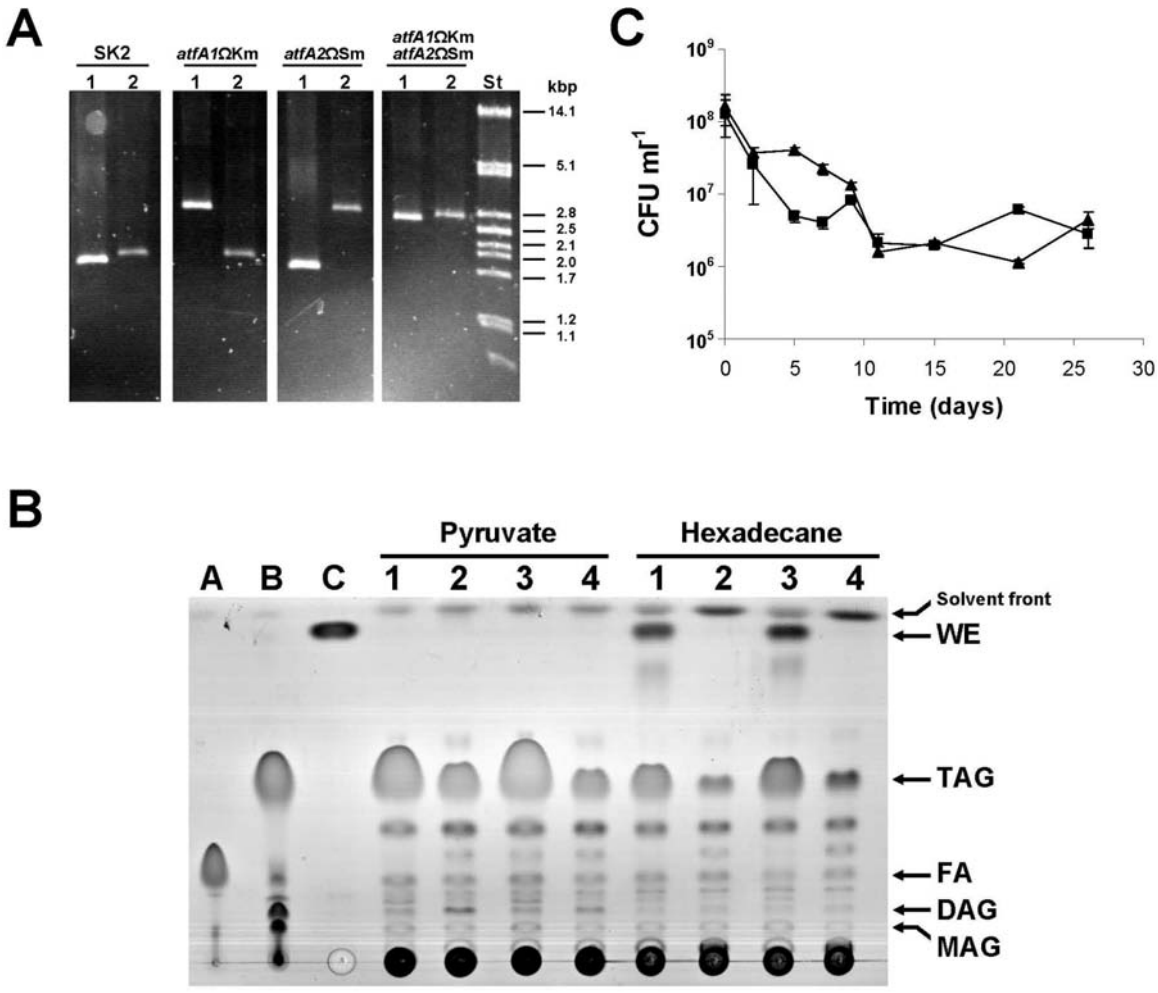
1 FIG. 2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1.7
1.2
1.1
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CFU ml-1
10^9
10^8
10^7
10^6
10^5
0 5 10 15 20 25 30
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TAG
FA
DAG
MAG

FIG. 3

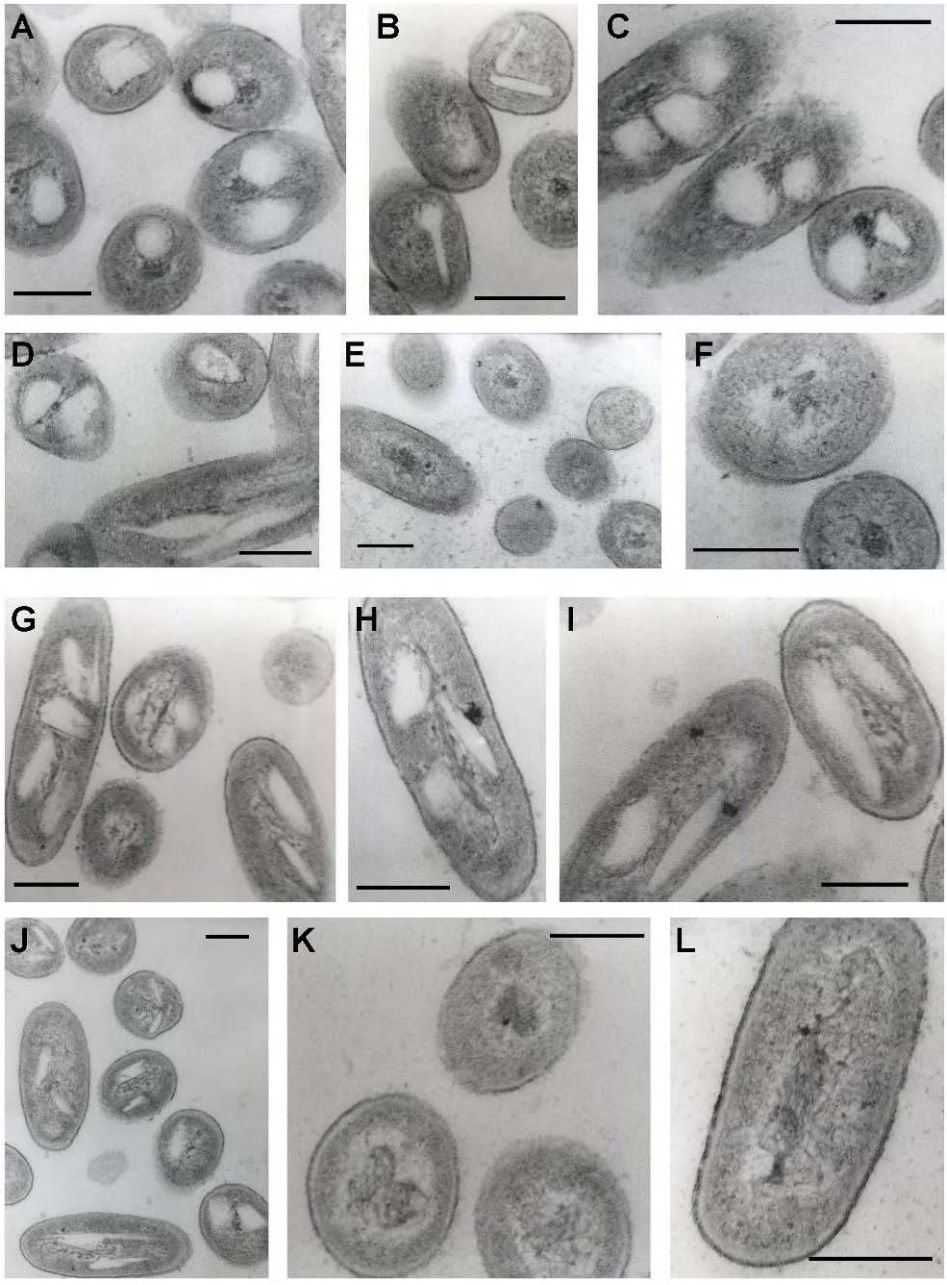


FIG. 4

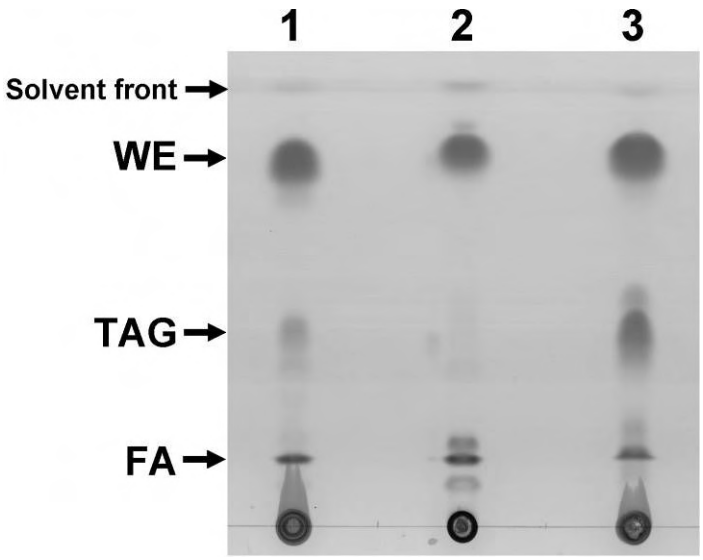
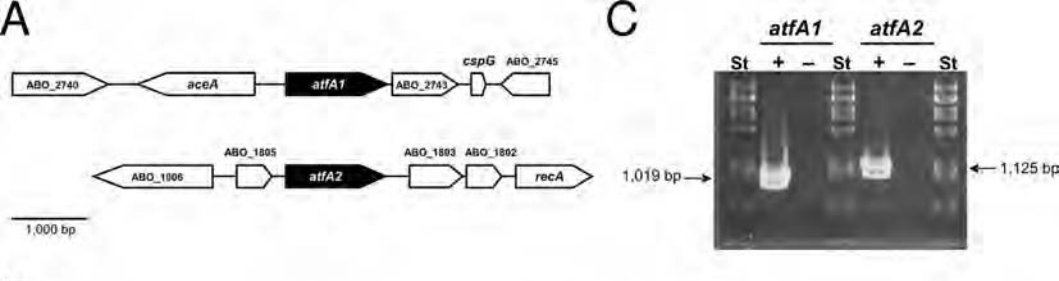


**Table S1**

# Putative acyltransferase genes within the *Alcanivorax borkumensis* SK2 genome

| Product Name | Start | End | Strand | Length | Gi | GeneID | Locus | Locus_tag |
|---|---|---|---|---|---|---|---|---|
| acyltransferase | 56321 | 58141 | + | 606 | 110832916 | 4213934 | - | ABO_0055 |
| acyltransferase | 83762 | 84301 | - | 179 | 110832940 | 4213441 | - | ABO_0079 |
| acyltransferase, putative | 751502 | 753295 | - | 597 | 110833538 | 4212673 | - | ABO_0677 |
| acyltransferase, putative | 1024717 | 1026672 | + | 651 | 110833778 | 4212819 | - | ABO_0917 |
| acyltransferase, putative | 1265055 | 1265699 | + | 214 | 110833973 | 4213192 | - | ABO_1112 |
| acyltransferase, putative | 1590189 | 1591082 | + | 297 | 110834258 | 4211772 | - | ABO_1397 |
| acyltransferase, putative | 1608404 | 1609228 | - | 274 | 110834271 | 4213262 | - | ABO_1410 |
| acyltransferase, putative | 2824856 | 2825746 | - | 296 | 110835353 | 4212601 | - | ABO_2492 |

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AtfA1    1 -MKALSQVDQLPLWLEKRQQPMHVGGLQLFSFPEGAGPKYVSELAQQMR-DYCHPVAPNQRDTRRL---GQYYWTRDKQFDIDNHFRHEALPKPGRIRE  95
AtfA2    1 MARKLSIMDSGWLMMETRETPMHVGGLALFAIPEGAPEDYVESIYRYLV-DVDSICRPFNQKIQSHLPLYLDATWVEDKNFDIDYHVRHSALPRPGHVRE  99

AtfA    93 LGIYISQSHSTLLDMAKPLWTCNIIEGIEGNRFAMVFKPHHAMVDGVAGMRLIERSLSHDVTPKSIVPPWCVEGKRAKKLREPKTGKIKKI-MSGIKSQL 191
AtfA1   96 LLSLVSASRSNLLDMERPMWEAHLIEGIRGRQPALVYKPHHSVMDGISAMRIASKTLSTDPSPREMAPAWAFNTKKRSLSLPSNPVDMASS-MARLTASI 194
AtfA2  100 LLALVSRLHAQRLDPSRPLWESYLIEGLEGNRPALVTKMHHSMVDGVAGMHLMQSRLATCAEDR-LPAPWSGEWDAEKKPRKSRGAAAANAGMKGTMNNL 198

AtfA   192 QATPTVIQKLSQTVFKDIG---RNPDHVGSPQAPCSILNQRVSSERRFAAQSFDLDRFRNLAKSLNVTINDVVLAVCSGALRAYLMSHNSLPSKPLIAMV 289
AtfA1  195 SKQAATVPGLAREVYKVTQKAKKDENYVSIFQAPDTILNNTITGSRRFAAQSFPLPRLKVLAKAYNCTINTVVLSMCGHALREYLISQHALPDEPLIAMV 294
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AtfA1  295 PMSLRQDDSTG--GNQIGKILANLGTHICDPANRLRVIHDSVEEAKSRPSQMSPEEILNFTALTMAPTGLNLLTGLAPEWR-APNVVISNIPGPKEPLYW 391
AtfA2  291 PVALRSADQAGEGGNAITTVQVSLGTHIAQPLNRLAAIQDSMKAVKSRLGDMQKSEIDVYTVLTNMPLSLGQVTGLSGRVSPMFNLVISNVPGPKETLHL 390

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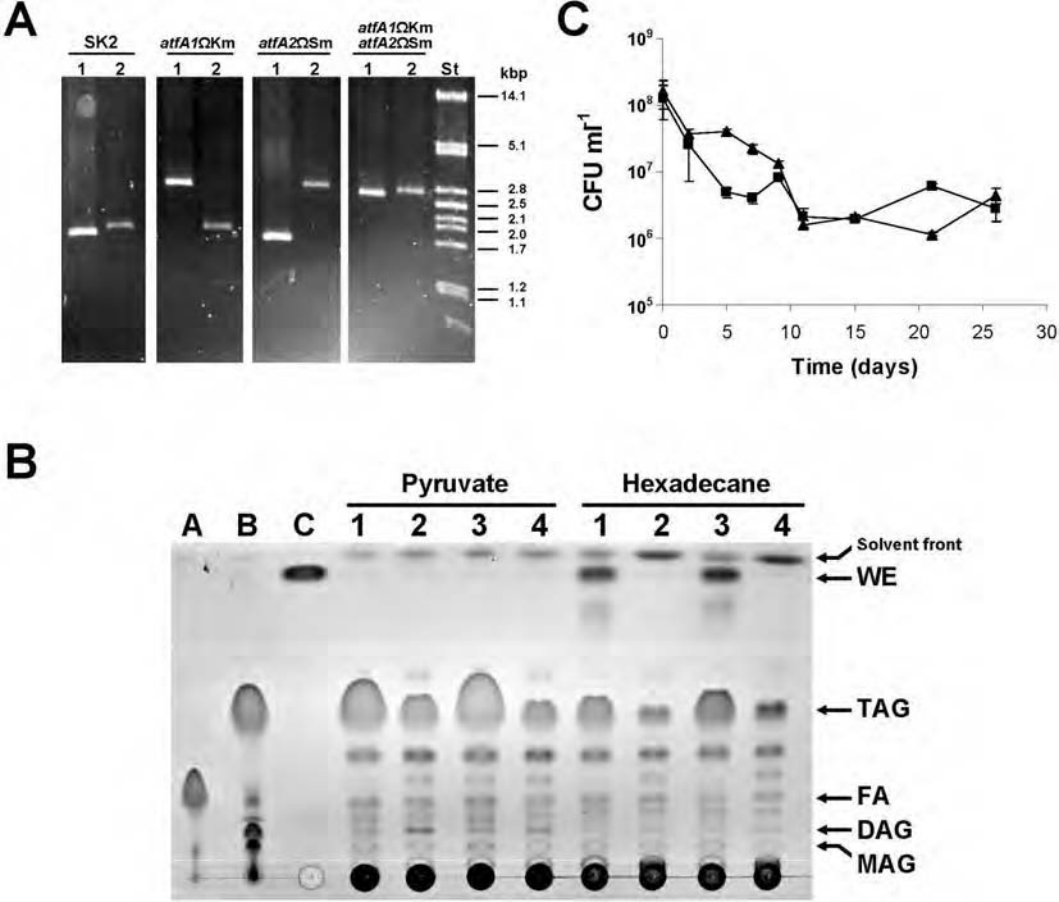
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kbp
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C
CFU ml-1
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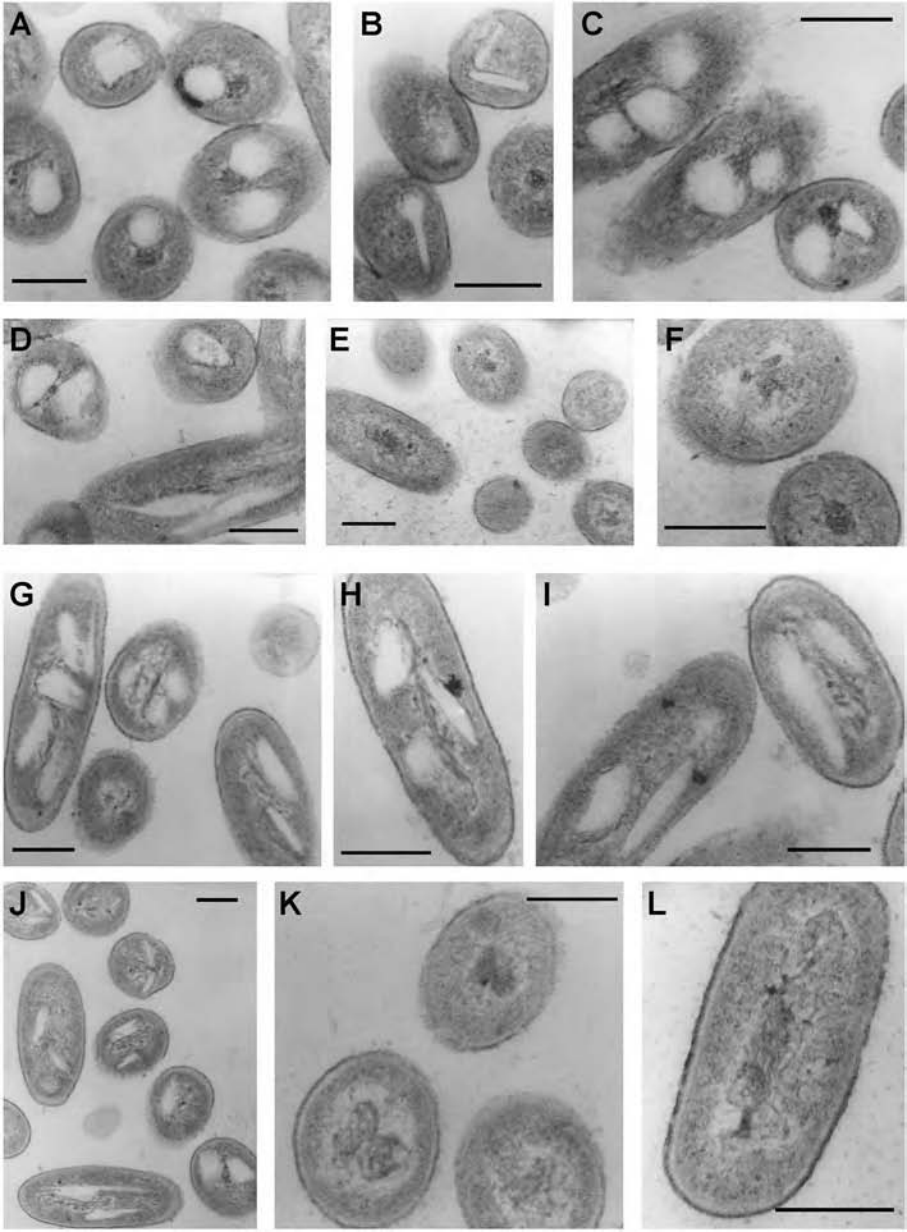
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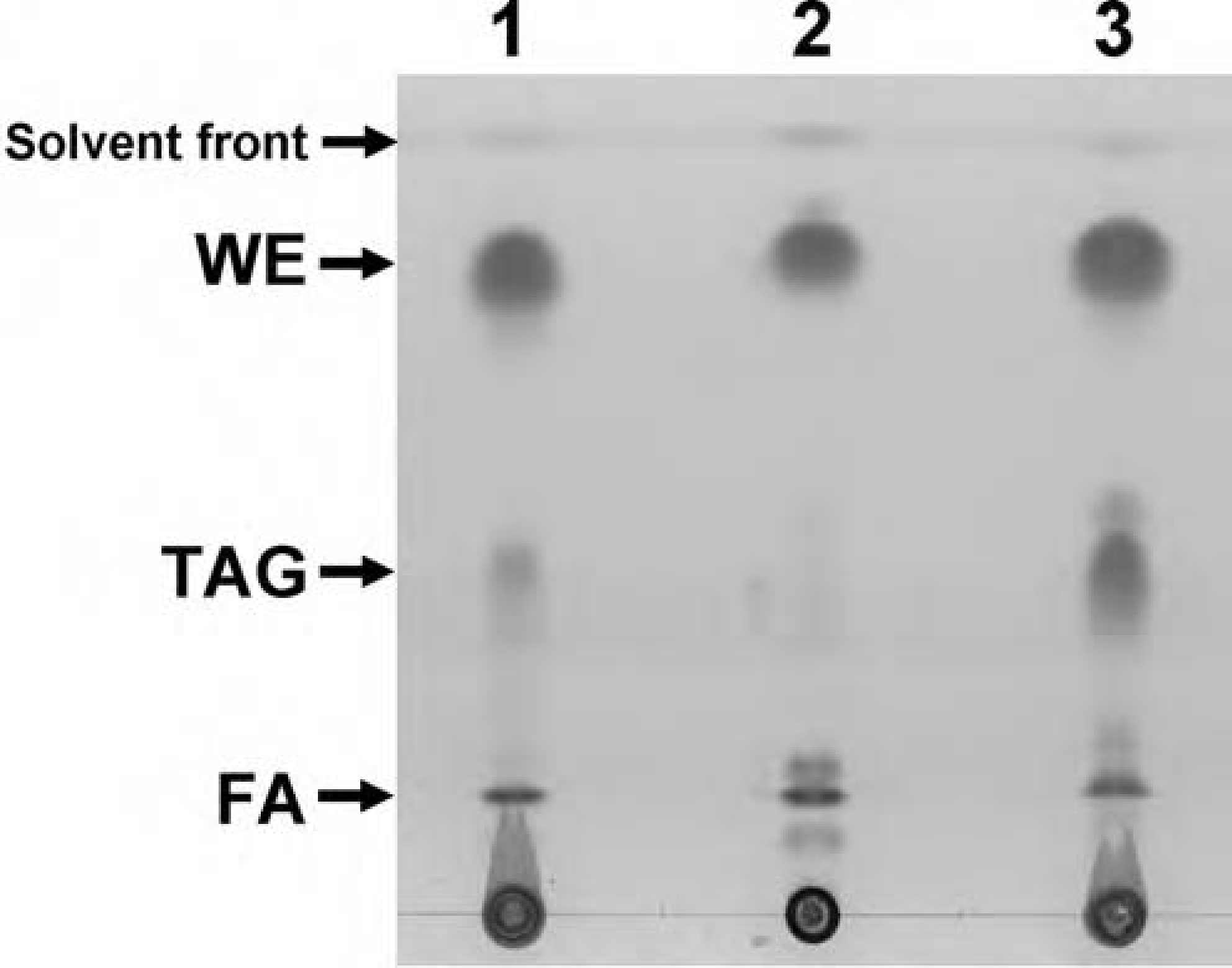
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