Mutation in a ‘tesB-like’ hydroxyacyl-CoA-specific thioesterase gene causes hyper-production of extracellular polyhydroxyalkanoates by *Alcanivorax borkumensis* SK2

Running title: Hydroxyacyl-CoA-specific thioesterase of *A. borkumensis*

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A novel mutant of the marine oil-degrading bacterium *Alcanivorax borkumensis* SK2, containing a mini-Tn5 transposon disrupting a ‘tesB-like’ acyl-CoA thioesterase gene, was found to hyper-produce polyhydroxyalkanoates (PHA), resulting in extracellular deposition of this biotechnologically important polymer, when grown on alkanes. The ‘tesB-like’ gene encodes a distinct novel enzyme activity which acts exclusively on hydroxylated acyl-CoAs, and thus represents a hydroxyacyl-CoA-specific thioesterase. Inactivation of this enzyme results in re-channeling of CoA-activated hydroxylated fatty acids, the cellular intermediates of alkane degradation, towards PHA production. These findings may open up new avenues for the development of simplified biotechnological processes for production of PHA as a raw material for the production of bioplastics.

*Alcanivorax borkumensis* strain SK2 is a cosmopolitan marine bacterium with a specialized metabolism adapted to the degradation of petroleum oil hydrocarbons, enabling it to degrade a wide range of hydrocarbons (26). *A. borkumensis* is usually the most abundant member of microbial communities that develop following an oil spill at sea, and is assumed to be globally one of the most important microbes involved in removing oil from marine environments (8). The genome of SK2 was recently sequenced and annotated (21). It is the best studied - and the paradigm - of the so-called hydrocarbonoclastic bacteria, a recently discovered group of oligotrophic marine microbes belonging to the *Gammaproteobacteria*, that utilize hydrocarbons but not most other common bacterial sources of carbon and energy.

In this study we identify and describe a new ‘tesB-like’ gene of *A. borkumensis* that encodes a novel hydroxyacyl-CoA-specific thioesterase. Acyl-CoA
thioesterases, that hydrolyse acyl-CoA molecules, have this far mainly been studied in
E. coli, which possesses two of such enzymes: (i) thioesterase I, encoded by the tesA
gene, cleaves C₁₂-C₁₈ acyl-CoA molecules (4); and (ii) thioesterase II, encoded by the
tesB gene, acts on C₆-C₁₈ acyl-CoA thioesters as well as on C₁₂-C₁₈ 3-hydroxyacyl-
CoA thioesters (3). Little is known about the exact physiological role of TesB protein
in the bacterial metabolism except that it releases free fatty acids, and at least in one
case, also hydroxylated fatty acids from the corresponding CoA-activated forms thus
producing free 3-hydroxyalkanoic acids (3-HAA) (27). CoA-activated hydroxylated
fatty acids in turn are cellular precursor intermediates for the synthesis of
polyhydroxyalkanoates, well-known bacterial storage compounds, which usually are
produced as insoluble intracellular granules by many microorganisms during times of
carbon surfeit (23), and they have long been explored as renewable resource for
biodegradable thermoplastics and biopolymers (2; 23; 17). We describe here that
disruption of the ‘tesB-like’ gene of A. borkumensis by a mini-Tn5 transposon causes
hyper-production and extracellular deposition of medium-chain length
polyhydroxyalkanoates (MCL-PHA) when growing on alkanes. Since commercial
exploitation of the biological production of PHA has this far been hampered by the
need for costly recovery of intracellularly stored granules from whole cells (11), the
present mutant allows to circumvent this costly recovery step, as large amounts of
PHA can simply be obtained from the culture medium.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A. borkumensis strain SK2 (DSM
No. 11573) is the wild type parental strain in all experiments. A mini-Tn5 mutant,
named C9 was generated by standard procedures using the mini-Tn5 Str/Sp element
SK2 wild type and C9 mutant strains were grown at 30°C in modified ONR7a medium (26) containing 0.27 g/l of NH₄Cl and either 1.5% (wt/vol) octadecane or 2% (wt/vol) pyruvate as carbon sources. *E. coli* strains DH5α (Invitrogen; Carlsbad, CA, USA) and Rosetta Blue DE3 (Novagen; Madison, Wisconsin, USA) used for cloning and expression studies were grown at 37°C in Luria-Bertani medium supplemented with kanamycin (50 µg/ml), or streptomycin (50 µg/ml) and/or chloramphenicol (34 µg/ml), where appropriate.

**Construction of a mini-Tn5 transposon library of *A. borkumensis* SK2.**

Transposon mutagenesis was based on the mini-Tn5 Str/Sp element constructed by de Lorenzo *et al.* (12). *A. borkumensis* SK2 was grown at 30°C on ONR7a medium until the stationary phase of growth and cells were centrifuged at 3200 g at 4°C. The donor strain *E. coli* (CC118 λ pir) and helper (HB101 λ pir) cultures of *E. coli* were grown overnight at 37°C on LB medium with either streptomycin or chloramphenicol respectively, washed with fresh LB and centrifuged at 3200 g at 4°C. The pellets of *A. borkumensis* and *E. coli* donor and helper strains were mixed in proportion 4:1:1 (by vol) and placed on a membrane filter on a plate with LB agar and salts (Na₂HPO₄×2H₂O – 0.45; NaNO₃ – 2.5; NaCl – 11.5; KCl – 0.38; CaCl₂×2H₂O – 0.7 g/l) and 2% (wt/vol) pyruvate as carbon and energy source. The plate was incubated for 24 hours at 30°C. The cells were then washed with 10 mM MgSO₄ and transconjugants were selected on ONR7a with 0.5% (wt/vol) pyruvate and 0.5% (wt/vol) acetate as carbon sources and nalidixic acid (10 µg/ml) and streptomycin as antibiotics as required.

**Inverse PCR.** The mini-Tn5 insertion sites of the selected mini-Tn5 mutants were determined by inverse PCR as described previously (15). Briefly, total DNA of the mutant was isolated and digested with *ClaI*, which does not cut within the mini-
Tn5 element. The resulting DNA fragments were circularized with DNA T4 ligase and the flanking regions of the inserted mini-Tn5 were amplified with two primers corresponding to the OTR End (GGC CGC ACT TGT GTA TAA GAG TCA G) and the 1TR End (GCG GCC AGA TCT GAT CAA GAG ACA G), respectively. The conditions for the PCR were: 94ºC 1.5 min; 48ºC 1 min; 70ºC 4 min, 30 cycles. The PCR products were gel-purified and used for automatic DNA sequencing with BigDye terminators on an ABI Prism 377 sequencer (AP Biosystems). To determine the precise site of transposon insertion, additional primers have been designed to read the flanking regions of the disrupted gene, i.e. 1086 (TTA CTG GCT TCG CAG GAA TGG) and intSM160 (CTT GGC ACC CAG CAT GCG CGA GCA GG).

Reverse transcription polymerase chain reaction (RT-PCR). In order to determine whether the two genes (ABO_1111 and ABO_1112) constitute an operon, RT-PCR was performed on DNase I-treated total RNA, extracted with Fast Blue RNA isolation kit (Qbiogene; Heidelberg, Germany) from a 10 ml of culture of SK2 grown to early stationary phase (OD600: 1.0) on either 2% (wt/vol) pyruvate or 1.5% (wt/vol) octadecane. Primers used were Oligo I (TAT GGT CAA AGT CAG GCG GTG) and Oligo II (CAC ATC CAA GCG CAA A GA CTG), specific for a 311-bp region spanning the 3’ end of ABO_1111 and the 5’ end of ABO_1112 (21). The same primers were also used for RT-PCR with RNA isolated from the C9 tesB-like::Tn5 mutant, in order to determine whether the mini-Tn5 mutation had a polar effect on the transcription of the downstream gene(s). RT-PCR was performed using the SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen), according to the instructions of the supplier. Briefly, the reaction mixture consisting of 2 µl of template RNA, 1 µl of a 10 mM dNTP mix, 1 µl of 2 µM primer Oligo II, and 6 µl of DEPS-treated water, was incubated at 65ºC for 5 min, placed on ice, then mixed with
a solution consisting of 2 µl 10xRT buffer, 4 µl of 25 mM MgCl₂, 2 µl of 0.1M dithiotreitol (DTT), and 1 µl of RNaseOUT Recombinant RNase inhibitor, incubated further at 42°C for 2 min, then 1 µl (50 units) of SuperScript II RT was added to each tube (except the RT control tubes), and incubation continued at 42°C for 50 min. The RT reaction was then stopped by raising the temperature to 70°C for 15 min. One µl of RNase H was then added and the mixture was incubated for 20 min at 37°C. Subsequent PCR amplification was performed under standard conditions, and the RT-PCR products were separated by electrophoresis on a 1.8% (wt/vol) agarose gel.

**PHA isolation and analysis of its monomer composition.** Bacteria were cultured in ONR7a medium with either 2% (wt/vol) of pyruvate or 1.5% (wt/vol) octadecane as carbon sources, on a rotary shaker (100 rpm) at 30°C until late stationary phase of growth. Cell pellets and supernatant fluids of the wild type were separated by centrifugation (60 min x 12,000 g). As C9 mutant cells could not entirely be separated from the culture media by centrifugation, total cell cultures of both the wild type and the C9 mutant were also included in the chemical analysis. The cell pellets, supernatants or total cell cultures were lyophilised, rinsed with ice-cold water, dried again overnight at 80°C, and stored at room temperature, until use. To quantify PHA, the polyesters were extracted from accurately weighed freeze-dried samples by Soxhlet extraction with hot chloroform (95°C) as described by Cromwick et al. (6).

Briefly, chloroform extracts were filtered through Whatman paper to remove cell debris, and then concentrated and added to cold methanol to precipitate PHA. The precipitated PHA was washed with methanol, dried and subjected to gel filtration to select only cell polymers with molecular weight higher than 100,000 Da, thus eliminating any contamination of the samples by glucolipids or free 3-HAA that are potentially also produced under these conditions. To determine the polyester content
and composition, 2 mg of purified PHA were incubated with a mixture of chloroform : methanol : sulphuric acid (1 : 0.85 : 0.15, by vol) for 2 h at 100°C to degrade PHA by methanolysis to its constituent 3-hydroxycarboxylic acid methyl esters (FAMEs). Distilled water (0.5 ml) was then added, tubes were shaken for 1 min and then the phases were allowed to separate. The organic phase was transferred into a vial and the FAMEs were analysed using a gas chromatograph-mass spectrometer (GC/MS, model Varian 3400CX, Varian Chromatography Systems, Sugar Land, TX, and VG Autospec spectrometer), equipped with a 30 m x 0.25 mm HP-5 (5% diphenyl and 95% dimethylpolysiloxane) fused silica capillary column; flow rate 1 ml/min (helium as carrier gas); sample input temperature to 230 °C at a rate of 8 °C/min; interface temperature 250 °C; ion source temperature 175 °C; electron impact mode 70 eV; scanning from 45 to 450 amu (atomic mass unit) at 0.5 s/scan. The degree of purity of the PHA samples used for analysis was about 99.5%, as no any additional peaks on GC chromatograms were observed (Fig. 1). Molecular weights were determined by gel permeation chromatography (GPC) using a Spectra Physics gel permeation chromatograph (Spectra-Physics). The GPC experiments were carried out in a Spectra Physics gel permeation chromatograph (Spectra-Physics) under the following conditions: column temperature, 50 °C; isocratic gradient; mobile phase, tetrahydrofuran (THF); flow rate, 1.0 ml/min; light scattering detector.

Cloning of tesB-like gene in E. coli and preparation of cell extracts for thioesterase assay. The gene ABO_1111 encoding for TesB-like acyl-CoA thioesterase was amplified using primers 1086F (5’–TTA CTG GCT TCG CAG GAA TGG–3’) and 1086R (5’–CTT GCT TAC CTA AAG TCC GCG–3’) and the resulting PCR product of 896 bp was cloned into the pCR2.1 TOPO cloning vector (Invitrogen). The cloned gene was then excised from the recombinant plasmid as
EcoRI fragment, and inserted into the EcoRI site of pCDFDuet-1 expression vector (Novagen), and the resulting construct was then transformed into competent *E. coli* DH5α cells (Invitrogen), selecting for transformants on LB containing streptomycin (50 µg/ml). The clones obtained were checked for correct orientation of the cloned gene, and positive plasmid constructs were then transformed into RosettaBlue™ DE3 competent cells and transformants were selected on streptomycin (50 µg/ml) and chloramphenicol (34 µg/ml). For expression and purification of the enzyme, overnight cultures of *E. coli* RosettaBlue™ DE3, harboring the tesB-like gene in the expression vector, were diluted 1:10 and grown at 37°C in LB liquid medium containing appropriate antibiotics until absorbance at 600 nm of 1.0 was reached. At that point, overexpression was induced by the addition of 1.0 mM of isopropyl thio-β-D-galactoside (IPTG), and after 6h of growth cells were harvested, washed with buffer A (50 mM potassium phosphate buffer, pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA) and stored at 4°C, until use. Approximately 0.5 g (wet weight) of *E. coli* cells expressing or not the tesB-like gene, were suspended in 1 ml of buffer A, supplemented with 200 µg of phenylmethylsulfonyl fluoride (PMSF), 5 µg DNase I grade II and 1 µg lysozyme per ml and cells were then disrupted by sonification for a total of 4 min (30 sec pulses, 1 min pauses) at 4°C in a W250 sonifier (Branson Schallkraft GmbH, Germany). Soluble cell fractions were obtained as supernatants after 30 min of centrifugation at 15,000 g at 4°C. The resulting supernatants were tested for thioesterase activity (see below). The total protein concentration was determined by the Bradford method using BSA as standard (5).

**Thioesterase assay.** The hydrolysis of acyl-CoAs and hydroxyacyl-CoAs by *E. coli* cell extracts containing or not the TesB-like enzyme was determined using a 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB)-based assay, as described elsewhere (28).
Briefly, reactions of the 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB)-based test were carried out in buffer A, and 5-thio-2-nitrobenzoate, produced from DTNB reacting with CoA released by hydrolysis from the acyl-CoA substrate, was monitored through its absorbance at 412 nm (molar extinction coefficient: 13,600 M$^{-1}$). A 1 ml reaction mixture contained 4 μM acyl-CoAs or hydroxyacyl-CoA (chain length in each case ranging from acetyl to decanoyl), 1 mM DTNB and 100 μl of crude cell extract containing 25 μg of total protein (obtained as described above) in a quartz cuvette of 1-cm light path length. One unit of enzymatic activity was defined as the amount of protein releasing 1 μmol of CoA per min. (R,S)-3-hydroxyacyl-CoAs (from 3-hydroxyhexadecyl-CoA to 3-hydroxydecanoyl-CoA) were synthesized as described by Rehm (18). (R,S)-3-acyl-CoAs were obtained from Sigma Chemicals Co. (St. Louis, Missouri). Quantification of the hydrolysis of hydroxyacyl-CoAs by extracts from *A. borkumensis* SK2 wild type and the C9 mutant strains was performed essentially as described above, using protein extracts of *A. borkumensis* cells cultured in ONR7a medium with either 2% (wt/vol) pyruvate or 1.5% (wt/vol) octadecane as carbon sources, on a rotary shaker (100 rpm) at 30ºC until late stationary phase of growth (conditions for disruption were as for *E. coli* cells).

**Electron microscopy.** For scanning electron microscopy, cells were grown on Permanox® slides (Nalge Nunc) in ONR7a containing either 1.5% (wt/vol) octadecane (slides covered with octadecane), or 2% (wt/vol) pyruvate, and 0.27 g/l NH$_4$Cl in liquid culture, and cells were harvested in their stationary phase of growth. Scanning electron microscopy was prepared and carried out as described previously (13).
RESULTS AND DISCUSSION

_A. borkumensis_ produces polyesters. For marine oil-degrading bacteria including _A. borkumensis_, oil pollution constitutes a temporary condition of carbon excess coupled with limiting nitrogen, i.e. a high carbon/nitrogen (C/N) ratio, which is precisely the condition that would prompt bacteria to embark on the formation of storage compounds such as polyhydroxyalkanoate (PHA) or other cellular storage substances (23). Such storage compounds permit oligotrophic bacteria like _A. borkumensis_ to survive less nutritious periods interceding those abundant in carbon sources e.g. during events of oil pollution. Since PHA is known to be a common polymeric storage compound, and since _A. borkumensis_ was reported earlier not only to express one of two putative phaC synthase genes (ABO_1418) under conditions of alkane excess (19), but also to be able to synthesize the monomeric PHA precursor compound 3-hydroxyalkanoate acid (3-HAA) (1), we inspected this organism’s ability to produce polyhydroxyalkanoates.

Chemical analysis by GC/MS of cell contents from _A. borkumensis_ SK2 wild type grown under conditions of C excess revealed that _A. borkumensis_ produces either medium-chain length PHA (MCL-PHA), consisting of 3-hydroxyacyl monomers of 6 to 12 carbon units, or polyhydroxybutyrate (PHB, 4 carbon units) depending on the carbon source used for growth (Fig. 1; Table 1). The composition of the polymer produced on alkanes corresponds well to the respective PHA monomer composition typical for _Pseudomonas_ species (24). The mean molecular weight of the polymer as determined by gel permeation chromatography was found to be around 280 kDa in both growth conditions tested (Table 1). However, although the amount of PHA produced from alkane (i.e under conditions of a high C:N ratio) was 3 times more (18 mg/l) than the amount of PHB produced during growth on pyruvate (6.5 mg/l), such
in absolute terms rather low concentrations are far below the amounts typically found for intracellular storage polymers in PHA- or PHB-storing bacteria (up to 1.6 g/l). The chemical analysis was also confirmed by NMR (data not shown). We conclude that, while *A. borkumensis* clearly has the genetic equipment to synthesize PHA and PHB polyesters, the wild type does so by producing only small amounts, suggesting that neither PHB nor PHA are likely to be major storage compounds in this bacterium, which instead probably employs other types of storage compounds to serve as carbon/energy source storage during periods of carbon/energy limitotrophy.

**Isolation of a mutant showing hyper-production and extracellular deposition of PHA.** In the course of the screening of a mini-Tn5 transposon library based on the mini-Tn5 Str/Sp element (12) applying Kolter’s assay (25) to look for biofilm deficient mutants, a number of mutants that failed to form a biofilm in 96-well microtitre plates were isolated (Sabirova et al., unpublished data). The Kolter assay is routinely being used for the detection of biofilm-deficient mutants, it involves growing the cells in 96-well microtitre PVC plates, staining biofilm-forming cells with crystal violet and finally washing out and measuring the retained dye with ethanol to quantify the degree of biofilm formation. Cells able to form biofilm normally produce a violet-stained circle at the air-water interface. In one of the biofilm deficient mutants, designated as C9 mutant, formation of biofilm was prevented by excessive production of secreted polymeric material, later identified as the bacterial storage compound polyhydroxyalkanoate (PHA) (see below). Scanning electron micrographs of SK2 wild-type and C9 mutant (Fig.2) cells grown on *Permanox*® hydrophobic slides covered with octadecane in ONR7a show that the mutant cells are embedded in a dense extracellular network of material, whereas the wild-type cells are not. As the cells were grown in excess of alkane source, i.e. under
conditions favouring the production of PHA storage material, we suspected the extracellularly deposited polymer to be PHA. To test this assumption, both wild type strain SK2 and mutant strain C9 were grown on ONR7a with 1.5% (wt/vol) octadecane as carbon and energy source, and polymer was extracted from each of the total cell cultures and was analysed as described in Materials and Methods section. As no PHA was detected in the culture supernatant of alkane-grown SK2 wild type cells, we conclude that essentially all of the PHA produced by the SK2 wild type on alkane was intracellular. As C9 mutant cells could not entirely be separated from the culture media by centrifugation (most likely due to the extracellular PHA tightly attached to them), we determined PHA yields in total cell culture. We found that in the C9 mutant grown on octadecane, the amount of PHA was almost 2.6 g/l, which is about 140× that produced by the SK2 parental strain grown on octadecane (0.018 g/l) (Table 1). PHA production was thus significantly higher in the C9 mutant strain than in the wild type under conditions where PHA precursor intermediates are made from alkane substrates.

The gene mutated in mutant strain C9 codes for a TesB-like acyl-CoA thioesterase. The site of insertion of the mini-Tn5 element in the C9 mutant was determined by inverse PCR as described elsewhere (15) and was found to be located between nucleotides 557 and 558 of the coding sequence (CDS) of the gene ABO_1111, which is annotated as coding for a putative TesB-like acyl-CoA thioesterase II (21). Analysis of the A. borkumensis genome sequence revealed that A. borkumensis possesses three different acyl-CoA thioesterases-encoding genes, namely tesA, tesB and the said tesB-like gene with the tesB and tesB-like genes being the closest homologues. The site of miniTn5 insertion in the tesB-like gene predicts a disruption of the gene’s function. Since the inverse PCR reaction produced only one
amplicon, we conclude that the phenotype of C9 results from the identified single transposition event. Inspection of the 3’ downstream region of ABO_1111 revealed the presence of a second CDS, ABO_1112, of 645 bp in length, which overlaps the last codon of ABO_1111 (Fig. 3A). The predicted encoded protein exhibits similarity to acylglycerolacyl transferase PlsC proteins of other bacterial species (amino acid identity/similarity: 41/55% to PlsC from P. aeruginosa PAO-1). The close proximity of ABO_1111 and ABO_1112 suggests that these two CDSs may form an operon. To determine whether the mutating transposon might exert a potential polar effect on the expression of the downstream ABO_1112 by preventing the expression of a potential operon-spanning single transcript, RT-PCR was employed (using primers Oligo I and Oligo II) to specifically amplify the 311 bp region spanning ABO_1111 and ABO_1112 junction (Fig. 3A). In both, the mutant and the wild type, we obtained the expected PCR product of approximately 311 bp. This confirms that ABO_1112 is well expressed also in the C9 mutant, either as a part of an operon with ABO_1111 with no polar effect of the insertion, or with ABO_1112 being transcribed from its own promoter. In any case, the amplified transcript indicative of ABO_1112 expression appears to be of comparable intensity in both the wild type and the C9 mutant (Fig.3B).

**Expression of TesB-like protein.** To determine the substrate specificity of the predicted TesB-like protein of A. borkumensis, we cloned the tesB-like gene (ABO_1111) into expression vector pCDFDuet-1 (Novagen) and expressed the gene in E.coli RosettaBlue™ DE3 competent cells (Novagen). E. coli crude extracts containing the expressed tesB-like gene product were then tested for enzymatic activity of the TesB-like protein. Acyl-CoA and (R,S)-3-hydroxyacyl-CoA were provided as substrates and the reaction products were analysed by a 5,5-ditio-bis(2-
nitrobenzoic acid) (DTNB)-assay as described in the Materials and Methods section, with *E. coli* harboring only vector pCDF as negative control. The data in Fig. 4A clearly demonstrate that the TesB-like enzyme efficiently hydrolyzes 3-hydroxyacyl-CoAs ranging from 3-hydroxyhexanoyl-CoA to 3-hydroxy-decanoyl-CoA, with clear preference for long-chain derivatives. By contrast, when the corresponding non-hydroxylated acyl-CoAs (ranging from hexanoyl to decanoyl) were provided as substrates, the TesB-like protein exhibited little ability to hydrolyze these non-hydroxylated acyl-CoA substrates (Fig. 4B). As thus crude extracts containing the cloned *A. borkumensis* tesB-like gene displays a very high ratio of hydroxyacyl-CoA to acyl-CoA-specific activity (approx. 500:1 for C_{10}-derivatives), we conclude that the tesB-like gene encodes a product which specifically acts on hydroxylated acyl-CoAs, and therefore can be named a hydroxyacyl-CoA-specific thioesterase. For future reference, we suggest to designate the tesB-like gene encoding the hydroxyacyl-CoA-specific thioesterase now tesB2, as opposed to the previously described tesB gene known to hydrolyze acyl-CoAs (Fig.4). Comparative measurement of 3-hydroxy-acyl-CoA thioesterase activity in *A. borkumensis* wild type and mutant strain C9 revealed that the mutation in tesB-like gene resulted in complete disruption of this enzymatic activity in the C9 mutant (Fig. 5), thus confirming that this novel enzymatic activity is indeed encoded by the tesB-like gene.

**Inactivation of the tesB-like gene channels 3-hydroxyacyl-CoA intermediates towards polyhydroxalkanoate production.** Amino acid similarity search of the mutated gene ABO_1111 against the entire *A. borkumensis* genome identifies an acyl-CoA thioesterase II protein, encoded by the tesB gene, as closest homologue. Two acyl-CoA thioesterases have been studied in *E. coli*: (i) acyl-CoA thioesterase I (encoded by the tesA gene), which is specific for C_{12}-C_{18} acyl-CoA
esters (4); and (ii) acyl-CoA thioesterase II (encoded by the \textit{tesB} gene), that cleaves C$_6$-C$_{18}$ acyl-CoA esters as well as C$_{12}$-C$_{18}$ 3-hydroxyacyl-CoA esters (3). Whereas TesA has been implicated in the hydrolysis of the thioester bond that links nascent fatty acids to the biosynthetic ACP-containing biosynthetic multienzyme complex, thus generating free fatty acids (10), little is known about the exact physiological role of TesB in the bacterial metabolism, except that it releases free fatty acids and at least in one case, hydroxylated fatty acids from their respective CoA-activated forms (27).

Since 3-hydroxyacyl-CoAs are the substrates of PHA-synthase in the formation of PHA, acyl-CoA thioesterases of the \textit{tesB}-encoded type releasing free 3-HAAs and PHA-synthase compete for the same intermediates as their substrates, namely 3-hydroxyacyl-CoAs. Hence inactivation of a \textit{tesB} gene encoding such an acyl-CoA thioesterase II able to also act on hydroxylated acyl-CoAs, or of a \textit{tesB}-like gene encoding such an enzyme acting specifically on hydroxylated acyl-CoAs only, as observed here in the case of \textit{A. borkumensis}, would in principle channel all 3-hydroxyacyl-CoA into PHA synthesis (Fig. 6), which can explain hyper-production of PHA as observed in \textit{A. borkumensis} C9 mutant deficient in the \textit{tesB}-like gene. Thus it appears that in \textit{A. borkumensis} the existence of two genes, \textit{tesB} and \textit{tesB}-like genes (\textit{tesB2}), reflects distinct functions of the TesB and TesB-like proteins, with the former acting specifically on non-hydroxylated, and the latter on hydroxylated acyl-CoAs exclusively. Indeed, a \textit{tesB}-encoded acyl-CoA thioesterase II unable to act on 3-hydroxyacyl-CoA has also been described for \textit{Rhodobacter spheroides} (20). A plausible explanation for the phenotype of mutant strain C9 therefore seems to be that the mutation inactivates the \textit{tesB}-like gene, whose protein product specifically acts on hydroxylated acyl-CoAs, and thus abolishes the release of free 3-HAA from 3-HAA-CoA, which leads to an increase of the pool of the PHA precursor molecule 3-
hydroxyacyl-CoA, and consequently to enhanced PHA formation. The potential metabolic pathways relevant to this scenario in *A. borkumensis* are depicted in Fig. 6.

The existence of a hydroxyacyl-CoA specific TesB-like thioesterase in *A. borkumensis* may be strongly linked to the alkane metabolism of this oil-degrading bacterium, such that this TesB-like thioesterase together with PHA synthase represent two “valve” enzymes allowing either to store the metabolic precursors in form of PHAs or to hydrolyze and possibly excrete them in the form of 3-HAAs. The latter have been shown to either possess biosurfactant properties themselves (7), or to be constituents of biosurfactants (22). In fact, *A. borkumensis* has been shown to produce biosurfactants of glycolipid nature in excessive amounts, some of them containing 3-hydroxy-alkanoic acid moieties (1), which should be highly advantageous during growth on alkane-containing oil spills.

To conclude, we report here a new enzyme found in the marine oil-degrading bacterium *A. borkumensis* which specifically hydrolyzes hydroxylated acyl-CoA, and that a mini-Tn5 mutation abolishing this 3-hydroxyacyl-CoA-specific thioesterase activity leads to hyper-production of extracellularly deposited PHA. This mutant’s ability to deposit overproduced PHA extracellularly provides an interesting starting point for studying the biological mechanisms by which PHA is translocated into the culture medium, in particular with regard to contrasting reports on mutants of other organisms that store overproduced PHA exclusively intracellularly (16). Apart from gaining insights into biological mechanisms, our findings present a novel system to potentially generate high yields of biotechnologically important PHA, which can easily be recovered from the culture medium and thus circumvents the need for costly procedures for the extraction of PHA granules from producer cells.
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REFERENCES


FIGURE LEGENDS

Figure 1. GC chromatograms of polyesters produced by *Alcanivorax borkumensis* SK2 and mutant C9 under different culture conditions.

Figure 2. Scanning electron micrograph images of *A. borkumensis* SK2 wild type and C9 mutant cells. SK2 wild type (a, c) and C9 mutant (b, d) cells, grown on Permanox® slides covered with octadecane in ONR7a medium containing 1.5% (wt/vol) octadecane and 0.27 g/l of NH₄Cl, featuring either the external, medium-exposed side of the biofilm (a, b), or the internal side of it, adjacent to the slide (c, d).

Figure 3. RT-PCR analysis of DNase I-treated RNA extracted from *A. borkumensis* SK2 and *tesB*-like acyl-CoA thioesterase mutant from a 10 ml of culture of SK2 grown to stationary phase (OD₆₀₀: 1.0) on either 2% (wt/vol) pyruvate or 1.5% (wt/vol) octadecane. Primers used were Oligo I and Oligo II, specific for a 311-bp region spanning the 3’ end of ABO_1111 and the 5’ end of ABO_1112. a) Organization of the operon and adjacent genes, location of the primers used, and predicted size of RT-PCR product. (b) RT-PCR products were obtained from total RNA extracted from SK2 and mutant C9, using primers Oligo I and Oligo II. Lanes: 1, 1-kb marker, 2, SK2 on pyruvate; 4, SK2 on octadecane; 6, C9 on pyruvate; 8, C9 on octadecane; lanes 3, 5, 7, 9 are corresponding negative controls (without reverse transcriptase).

Figure 4. Enzymatical hydrolysis of *(R,S)*-3-hydroxyacyl-CoAs (a) and acyl-CoAs (b) by crude extracts of *E. coli* harboring as control only vector pCDF (○) or the
recombinant insertion construct pCDF::tesB-like (●). Data given are means with
standard deviation of three independent culture samples and three independent assays.
The specific activity of crude extract of *E. coli* harboring only vector pCDF was lower
than 0.025 U/mg for all substrates tested, which is in the range of previously
published data.

**Figure 5.** Enzyme hydrolysis of (R,S)-3-hydroxyacyl-CoAs by *A. borkumensis* wild
type and its C9 mutant. 3-Hydroxy-dodecanoyl-CoA was the substrate for enzyme
determination. Assay was performed as described in Materials and Methods section
using 100 µl of crude cell extracts containing 25 µg of total protein. Activity is shown
as the amount of protein releasing 1 nmol of CoA per min. Values represent the
average of three determinations ± the standard deviation.

**Figure 6.** Suggested pathway of PHA biosynthesis in *A. borkumensis* SK2 and mutant
strain C9 grown on hydrocarbons. Hydrocarbons are degraded via terminal oxidation
to produce free fatty acids, which are then activated by an acyl-CoA synthase and
subjected to β-oxidation. The (S)-3-OH-acyl-CoAs produced by β-oxidation are
isomerised into (R)-3-OH-acyl-CoAs by the action of an isomerase. (R)-3-OH-acyl-
CoAs produced during β-oxidation are converted to either 3-hydroxyalkanoic acids
(3-HAA) through the action of TesB-like acyl-CoA thioesterase, or to
polyhydroxyalkanoate (PHA) through the action of PhaC synthase. The mutation in
the TesB-like acyl-CoA thioesterase abolishes production of free 3-HAA and
channels (R)-3-OH-acyl-CoAs exclusively into PHA synthesis.
FIGURE 1.

FIGURES AND TABLES
FIGURE 3.

A

phosphoglycerol transferase  tesB-like acyl-CoA thioesterase  acyltransferase  histidine kinase  ACBP

B

Oligo I  Oligo II

311 bp (WT and mutant)

1  2  3  4  5  6  7  8  9  10

500 bp

300 bp
FIGURE 4.
FIGURE 5.

Enzyme activity (nmol/mg min)

WT pyruvate | C9 pyruvate | WT alkane | C9 alkane

20

30

40

50

60
FIGURE 6.

Alkanes

\[ R \text{OCoA} \]

β-Oxidation

\[(S)-3-OH- \text{Acyl-CoA}\]

\[ R \text{O} \]

\[ (R)-3-OH- \text{Acyl-CoA}\]

3-HAA synthesis

3-Hydroxyalkanoic acid

PHA synthesis

Polyhydroxyalkanoate
**TABLE 1.** Polyhydroxyalkanoate (PHA) production in SK2 wild type and C9 mutant grown on different carbon sources.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>PHA yield (mg/L)</th>
<th>Monomer composition of hydroxyalkanoates (mol%)</th>
<th>Mw (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C₄  C₅  C₆  C₈  C₁₀  C₁₂</td>
<td></td>
</tr>
<tr>
<td>SK2 wild type</td>
<td>Pyruvate</td>
<td>6.5 ± 1.2</td>
<td>100  --  --  --  --  --</td>
<td>279 ± 23</td>
</tr>
<tr>
<td>SK2 wild type</td>
<td>Octadecane</td>
<td>18.0 ± 3.8</td>
<td>--  --  2 ± 0.3  20 ± 2.5  48 ± 3.7  30 ± 2.1</td>
<td>277 ± 38</td>
</tr>
<tr>
<td>C9 mutant</td>
<td>Pyruvate</td>
<td>112 ± 16.8</td>
<td>98 ± 0.4  2 ± 0.1  --  --  --  --</td>
<td>352 ± 16</td>
</tr>
<tr>
<td>C9 mutant</td>
<td>Octadecane</td>
<td>2560 ± 165.1</td>
<td>--  --  4 ± 0.2  18 ± 2.0  37 ± 2.5  39 ± 1.8</td>
<td>350 ± 42</td>
</tr>
</tbody>
</table>

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a To quantify PHA, the polyesters were extracted with chloroform as described in Materials and Method section and accurately weighed after gel permeation and GC analysis. b The molar fraction of polyester was calculated by gas chromatography according to the area of the peak of 3-hydroxycarboxylic acid methyl esters (FAMEs) obtained after methanolysis. Pure FAMEs were used for calibration. c Molecular weight of polymer, means as determined by gel permeation chromatography (GPC) calibrated with polystyrene. d not detected. In all cases data are means ± standard deviation (two independent cultures subject to three independent analyses).