Sabirova, J.S., Ferrer, M., Lünsdorf, H., Wray, V., Kalscheuer, R., Steinbüchel, A., Timmis, K.N., Golyshin, P.N.
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(2006) Journal of Bacteriology, 188 (24), pp. 8452-8459.

Mutation in a '*tes*B-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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Keywords: *Alcanivorax borkumensis*, acyl-CoA thioesterase, TesB, PHA, PHB, polyhydroxyalkanoates.

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A novel mutant of the marine oil-degrading bacterium *Alcanivorax borkumensis* SK2, containing a mini-Tn*5* 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 '*tes*B-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 C_{12}-C_{18} acyl-CoA molecules (4); and (ii) thioesterase II, encoded by the *tesB* gene, acts on C_6-C_{18} acyl-CoA thioesters as well as on C_{12}-C_{18} 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-Tn*5* mutant, named C9 was generated by standard procedures using the mini-Tn*5* Str/Sp element

(12). SK2 wild type and C9 mutant strains were grown at 30ºC in modified ONR7a medium (26) containing 0.27 g/l of NH_4Cl 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-Tn*5* transposon library of *A. borkumensis* SK2. Transposon mutagenesis was based on the mini-Tn*5* Str/Sp element constructed by de Lorenzo *et al*. (12). *A. borkumensis SK2* was grown at 30ºC 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 37ºC on LB medium with either streptomycin or chloramphenicol respectively, washed with fresh LB and centrifuged at 3200 g at 4ºC. 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 ($Na_2HPO_4 \times 2H_2O$ – 0.45; $NaNO_3$ – 2.5; NaCl – 11.5; KCl – 0.38; $CaCl_2 \times 2H_2O$ – 0.7 g/l) and 2% (wt/vol) pyruvate as carbon and energy source. The plate was incubated for 24 hours at 30ºC. The cells were then washed with 10 mM $MgSO_4$ 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-Tn*5* 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-

Tn*5* element. The resulting DNA fragments were circularized with DNA T4 ligase and the flanking regions of the inserted mini-Tn*5* 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 (OD_{600}: 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 AGA 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*::Tn*5* mutant, in order to determine whether the mini-Tn*5* 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 $MgCl_2$, 2 µl of 0.1M dithiotreitol (DTT), and 1 µl of RNaseOUT Recombinant RNase inhibitor, incubated further at 42ºC 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 42ºC for 50 min. The RT reaction was then stopped by raising the temperature to 70ºC for 15 min. One µl of RNase H was then added and the mixture was incubated for 20 min at 37ºC. 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 30ºC 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 80ºC, 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 (95ºC) 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 100ºC 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 chromotograph-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 ºC at a rate of 8 ºC/min; interface temperature 250 ºC; ion source temperature 175 ºC; 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 ºC; 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

*Eco*RI fragment, and inserted into the *Eco*RI 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 (50μg/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 4ºC, 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_4Cl 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-Tn*5* transposon library based on the mini-Tn*5* 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-Tn*5* 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 miniTn*5* 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 c*rude 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 polyhydroxyalkanoate 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 *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 *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 *tesB* gene encoding such an acyl-CoA thioesterase II able to also act on hydroxylated acyl-CoAs, or of a *tesB*-like gene encoding such an enzyme acting specifically on hydroxylated acyl-CoAs only, as observed here in the case of *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 *A. borkumensis* C9 mutant deficient in the *tesB*-like gene. Thus it appears that in *A. borkumensis* the existence of two genes, *tesB* and *tesB*-like genes (*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 tesB-encoded acyl-CoA thioesterase II unable to act on 3-hydroxyacyl-CoA has also been described for *Rhodobacter spheroides* (20). A plausible explanation for the phenotype of mutant strain C9 therefore seems to be that the mutation inactivates the *tes*B-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-Tn*5* 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 extacellularly 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.

ACKNOWLEDGEMENTS

This research was supported by grants from the German Ministry for Education and Research (BMBF) and European Community Project MERG-CT-2004-505242 "BIOMELI". JS is grateful to Dr. S. Lang (Technical University of Braunschweig), who was the first to give us a hint that the extracellular polymer might be PHA. JS also thanks Angelika Arnscheidt for technical assistance. MF thanks the Spanish Ministerio de Ciencia y Tecnología (Ramón y Cajal contract). K.N.T. thanks Fonds der Chemischen Industrie for generous support.

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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 NH_4Cl, 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 (OD_{600}: 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 ß-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.

FIGURES AND TABLES

FIGURE 1.

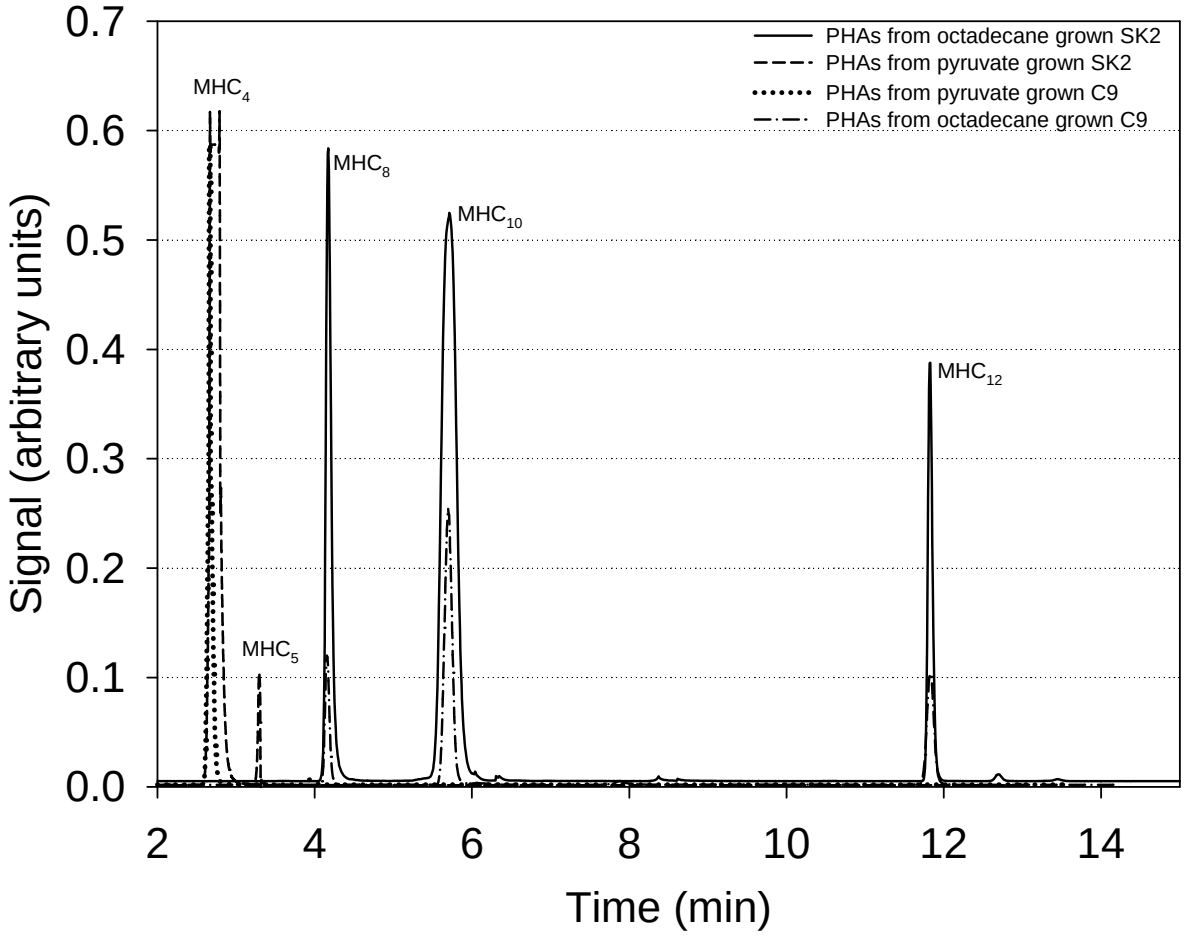


FIGURE 2.

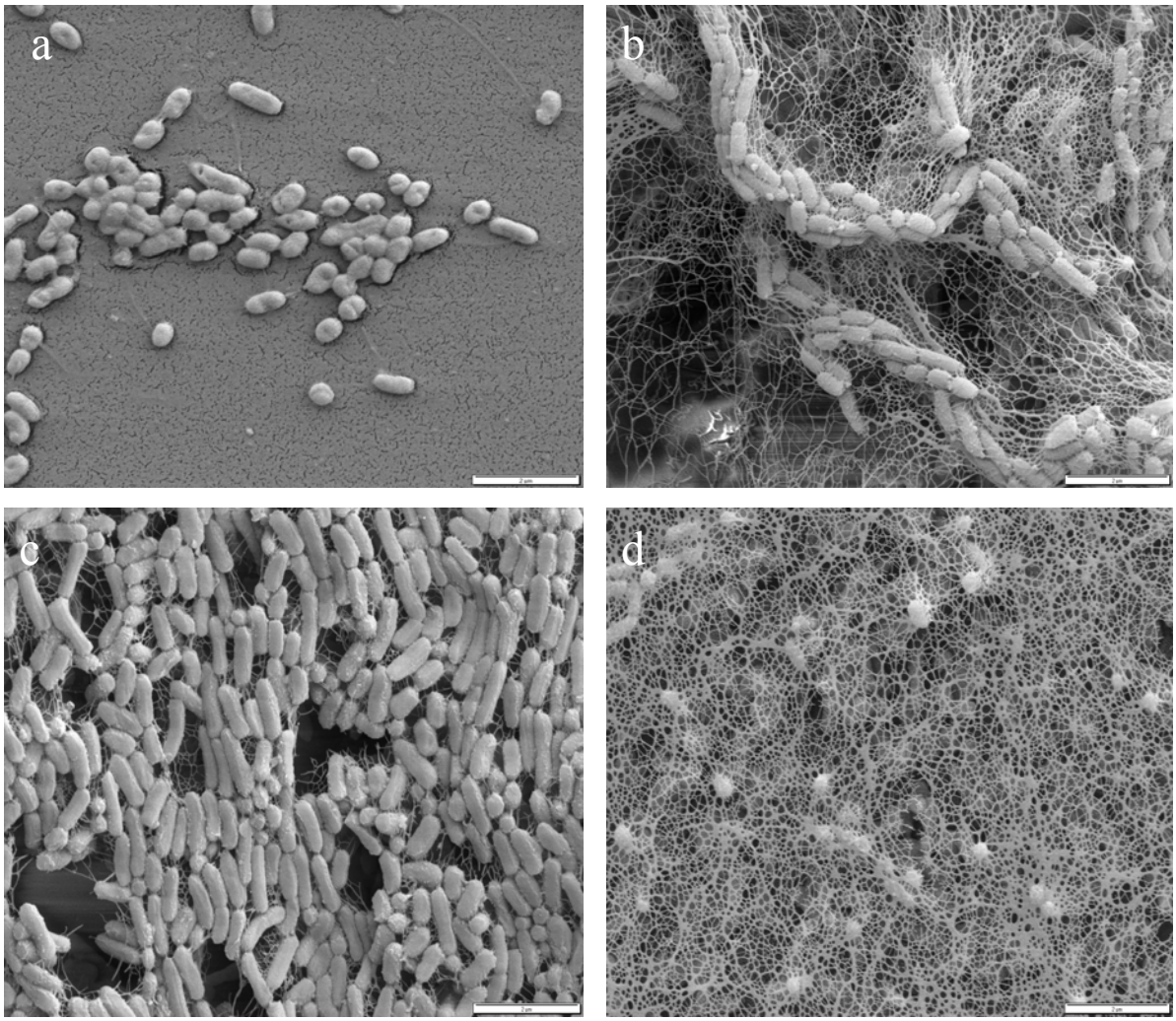
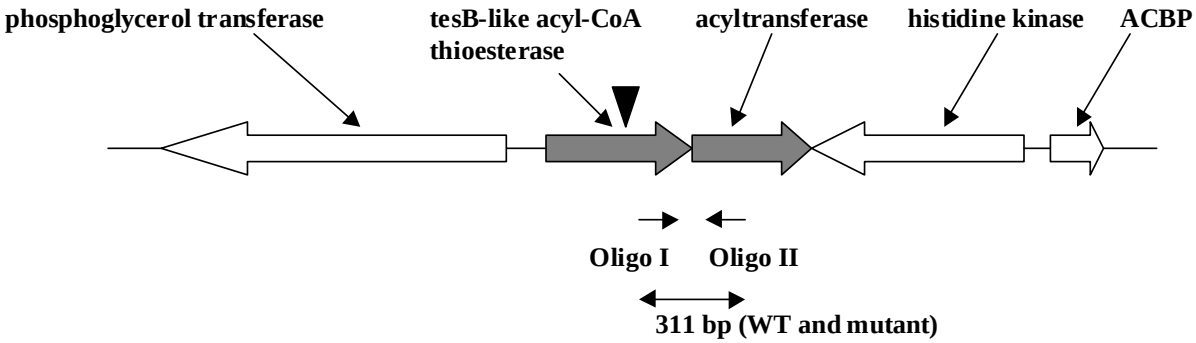


FIGURE 3.

A


phosphoglycerol transferase
tesB-like acyl-CoA thioesterase
acyltransferase
histidine kinase
ACBP
Oligo I
Oligo II
311 bp (WT and mutant)

B

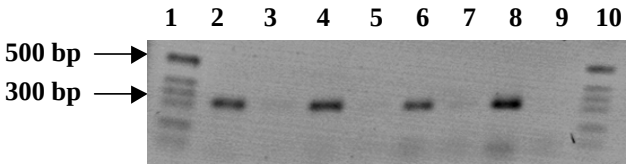

1 2 3 4 5 6 7 8 9 10
500 bp
300 bp

FIGURE 4.

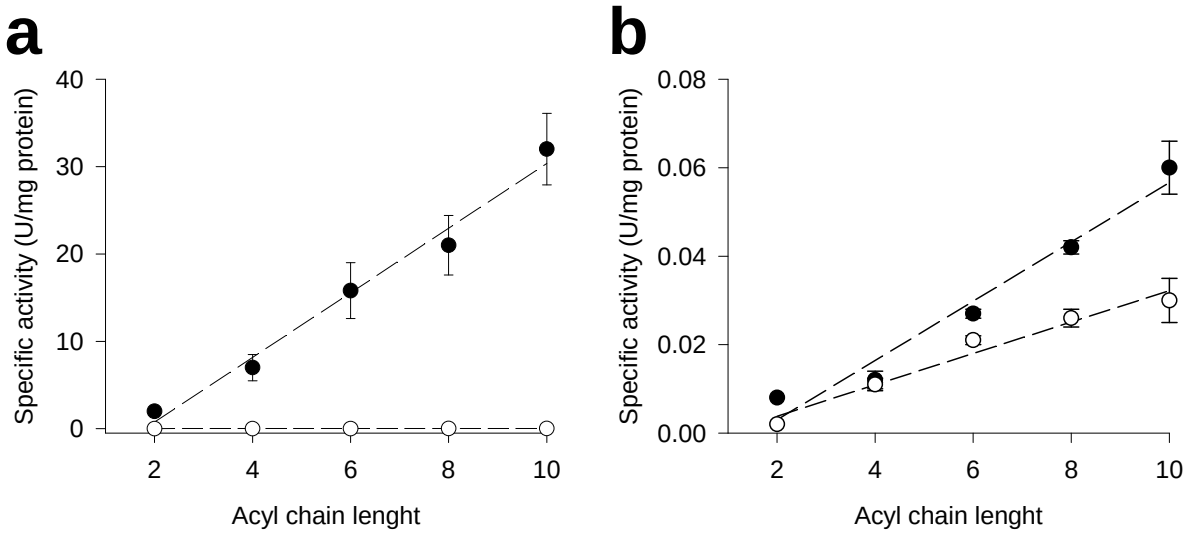

a
Specific activity (U/mg protein)
40
30
20
10
0
2
4
6
8
10
Acyl chain lenght
b
Specific activity (U/mg protein)
0.08
0.06
0.04
0.02
0.00
2
4
6
8
10
Acyl chain lenght

FIGURE 5.

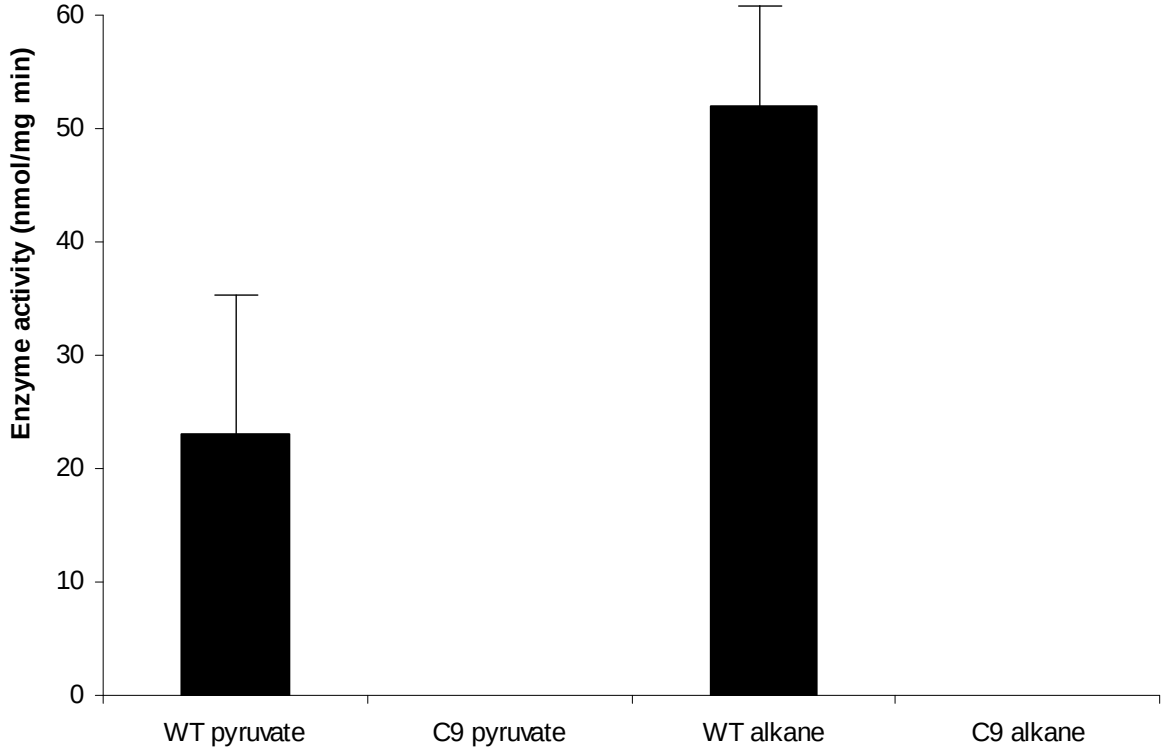

Enzyme activity (nmol/mg min)
60
50
40
30
20
10
0
WT pyruvate
C9 pyruvate
WT alkane
C9 alkane

FIGURE 6.

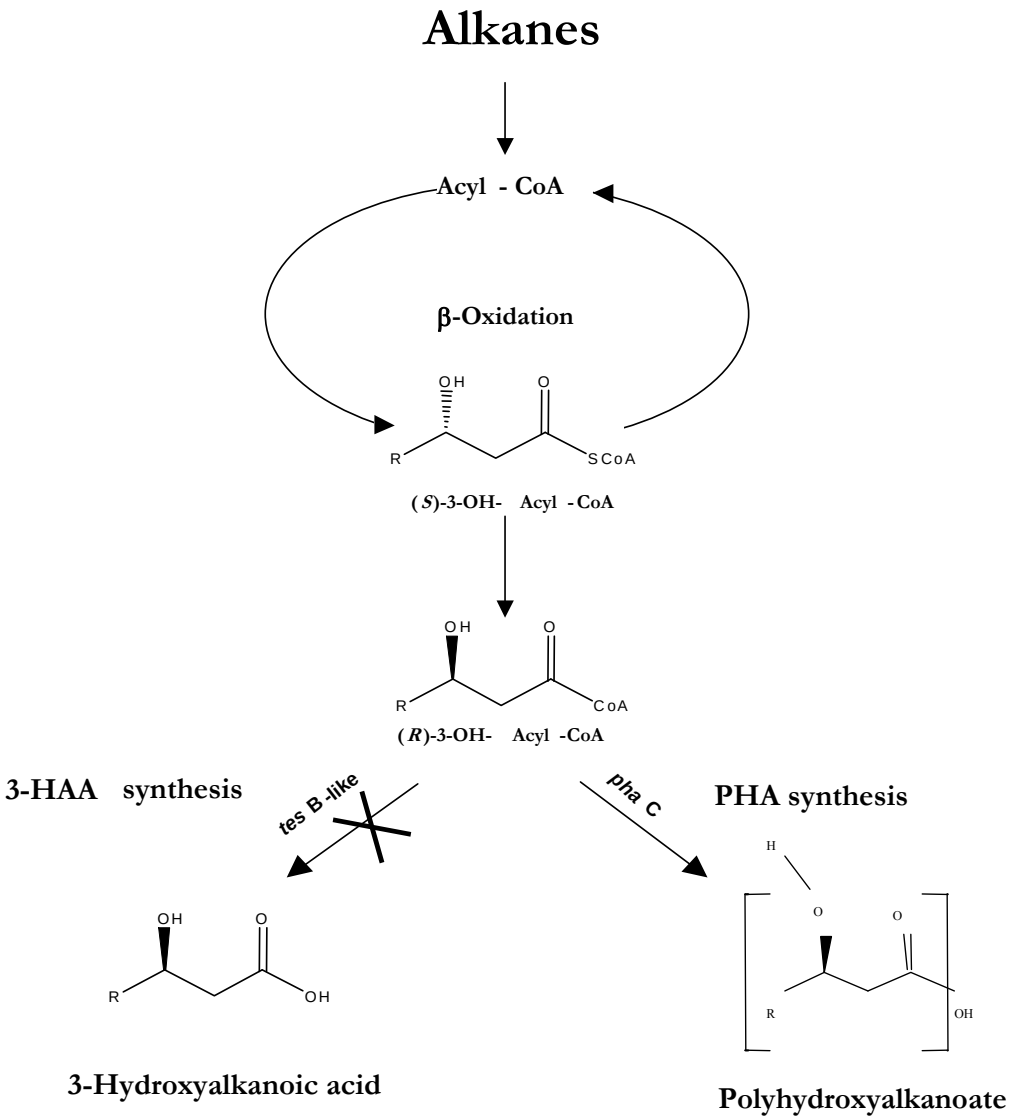

Alkanes
Acyl - CoA
β-Oxidation
OH
O
R
SCoA
(S)-3-OH- Acyl -CoA
OH
O
R
CoA
(R)-3-OH- Acyl -CoA
3-HAA synthesis
tes B -like
pha C
PHA synthesis
OH
O
R
OH
H
O
O
R
OH
3-Hydroxyalkanoic acid
Polyhydroxyalkanoate

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

Strain	Substrate	PHA yield (mg/L)[a]	Monomer composition of hydroxyalkanoates (mol%)[b]						Mw (kDa)[c]
			C_4	C_5	C_6	C_8	C_{10}	C_{12}	
SK2 wild type	Pyruvate	6.5 ± 1.2	100	–[d]	–	–	–	–	279 ± 23
SK2 wild type	Octadecane	18.0 ± 3.8	–	–	2 ± 0.3	20 ± 2.5	48 ± 3.7	30 ± 2.1	277 ± 38
C9 mutant	Pyruvate	112 ± 16.8	98 ± 0.4	2 ± 0.1	–	–	–	–	352 ± 16
C9 mutant	Octadecane	2560 ± 165.1	–	–	4 ± 0.2	18 ± 2.0	37 ± 2.5	39 ± 1.8	350 ± 42

[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).