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(54) **EXTRACELLULAR
POLYHYDROXYALKANOATES PRODUCED
BY GENETICALLY ENGINEERED
MICROORGANISMS**

(30) **Foreign Application Priority Data**

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(57) **ABSTRACT**

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(22) Filed: **Jan. 7, 2014**

Related U.S. Application Data

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30, 2010, now Pat. No. 8,623,632, filed as application
No. PCT/EP2006/007888 on Aug. 9, 2006.

The present invention is in the field of biosynthesis of polyhydroxyalkanoates (PHA). The invention relates to a genetically engineered microorganism having at least one gene involved in the metabolism, preferably in the production, of polyhydroxyalkanoates (PHA). This microorganism is useful in commercial production of polyhydroxyalkanoates. The present invention further relates to a method for the production of polyhydroxyalkanoates (PHA).

PHA production in *A.borkumensis* SK2 and the mutant strain on either 2% pyruvate or 1.5% octadecane as carbon sources.

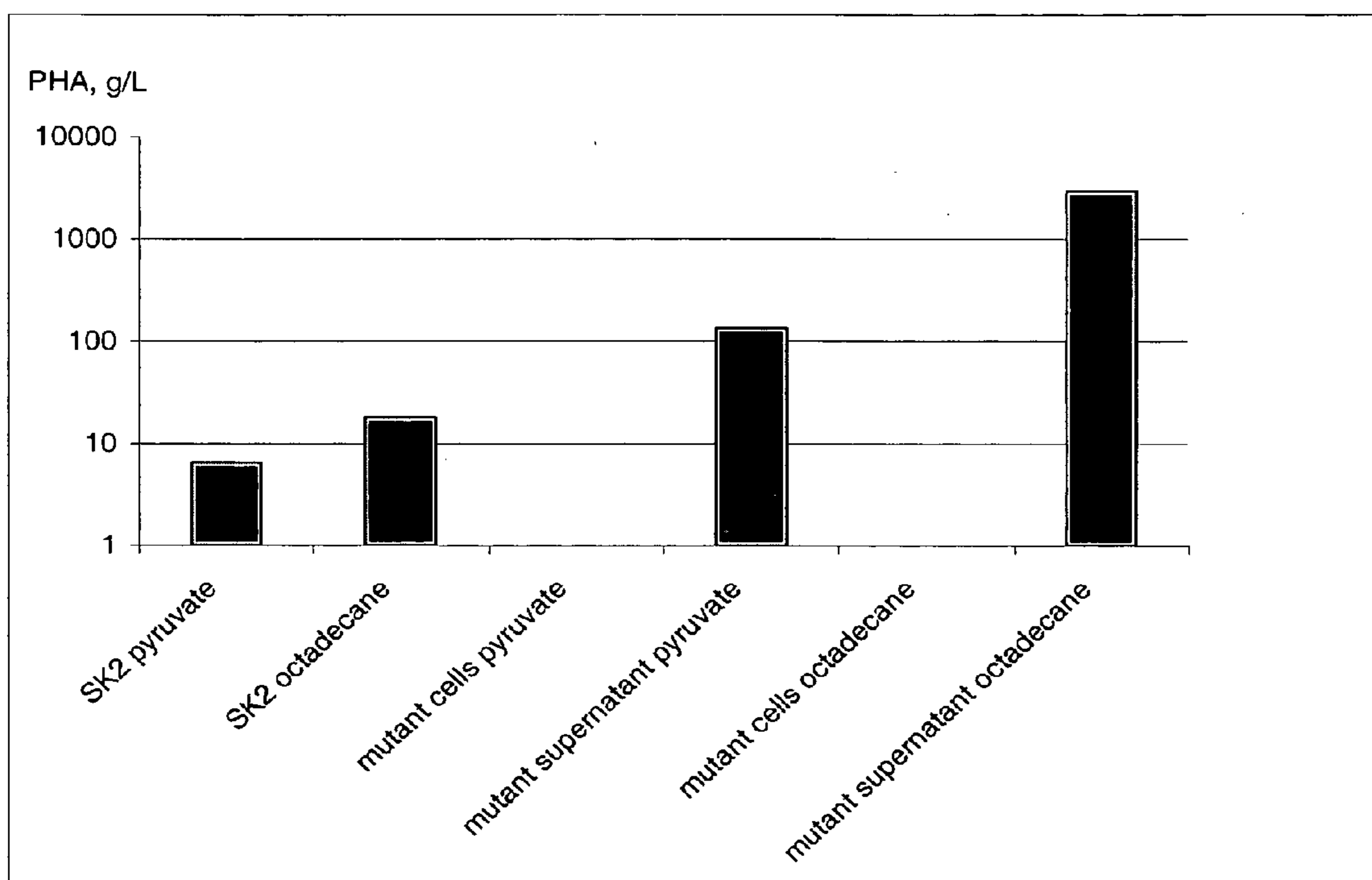


Figure 1

Electron microscopy images of the ultrathin sections of *A.borkumensis* SK2

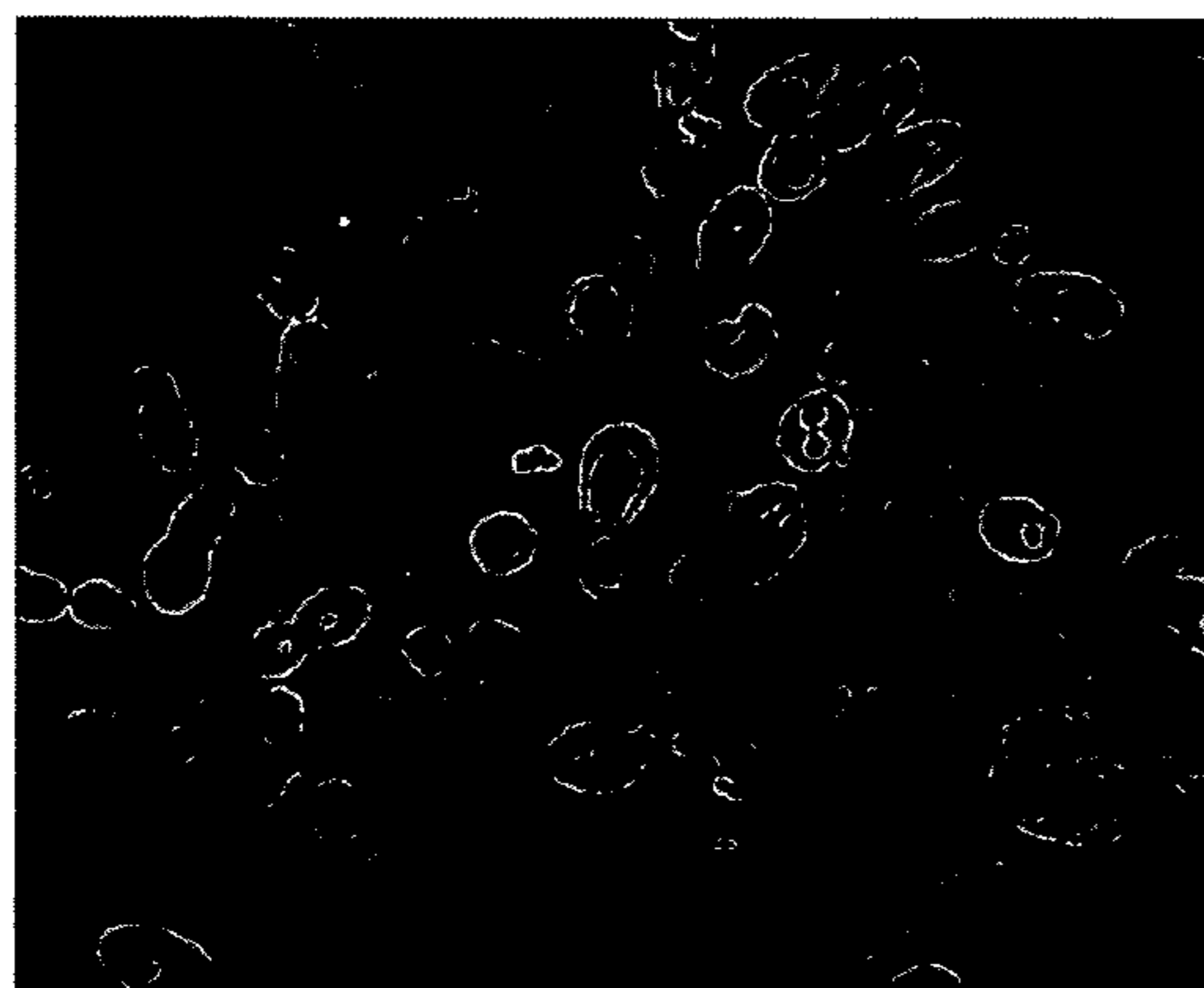


Figure 2

Scanning electron microscopy images (A and B) and electron microscopic images of the shadow-casted cells (C and D) of the wild type (left) and the mutant (right) prepared as it is described in the Materials and Methods. Cells were cultivated in ONR7a containing 1.5% (w/v) octadecane and 0.27 g/l of NH_4Cl (storage conditions) and were harvested in the stationary phase of growth..

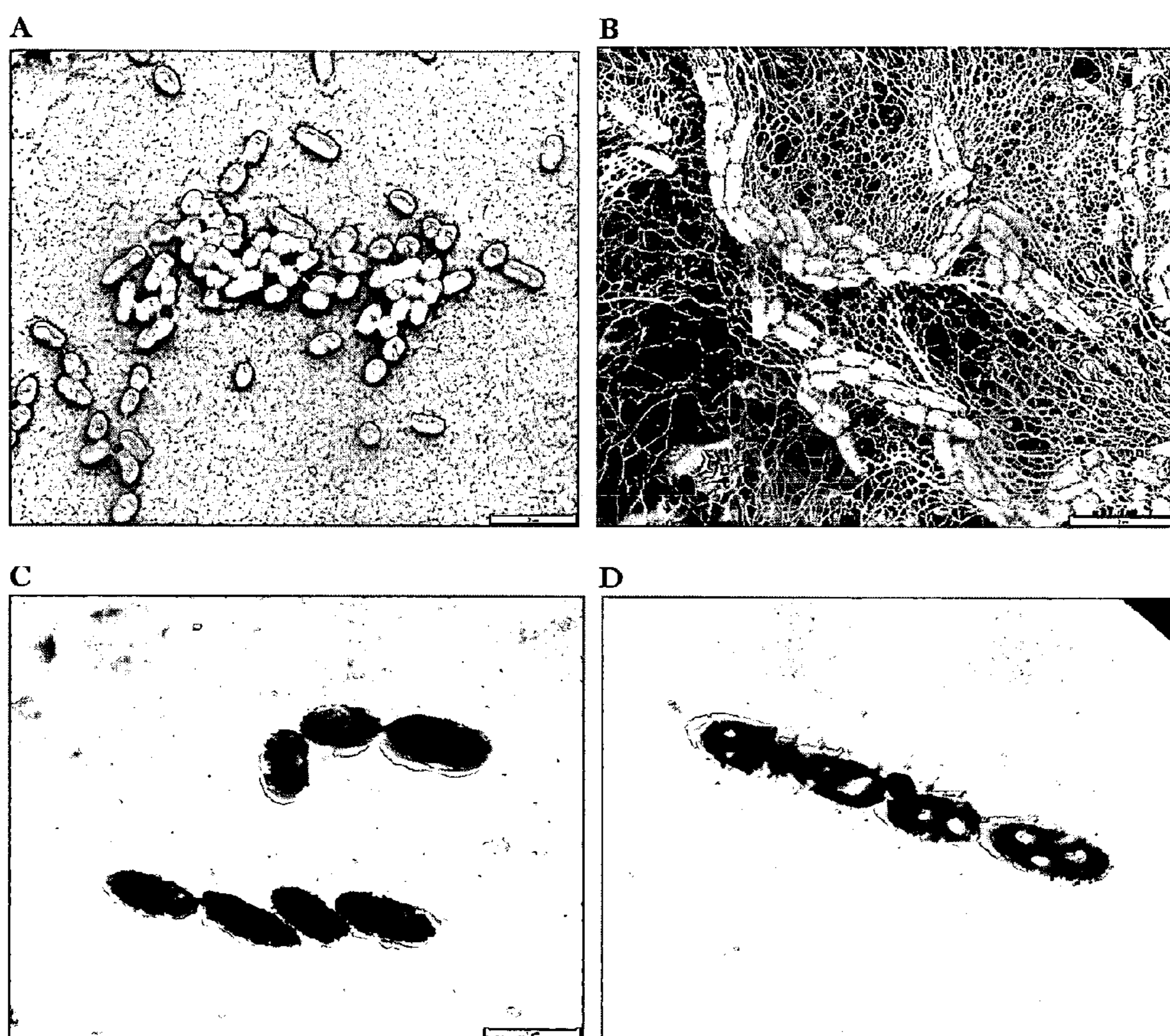


Figure 3

Growth characteristics of *A.borkumensis* wild type and mini-Tn5 mutant grown on pyruvate or octadecane under the conditions of high C:N ratio.

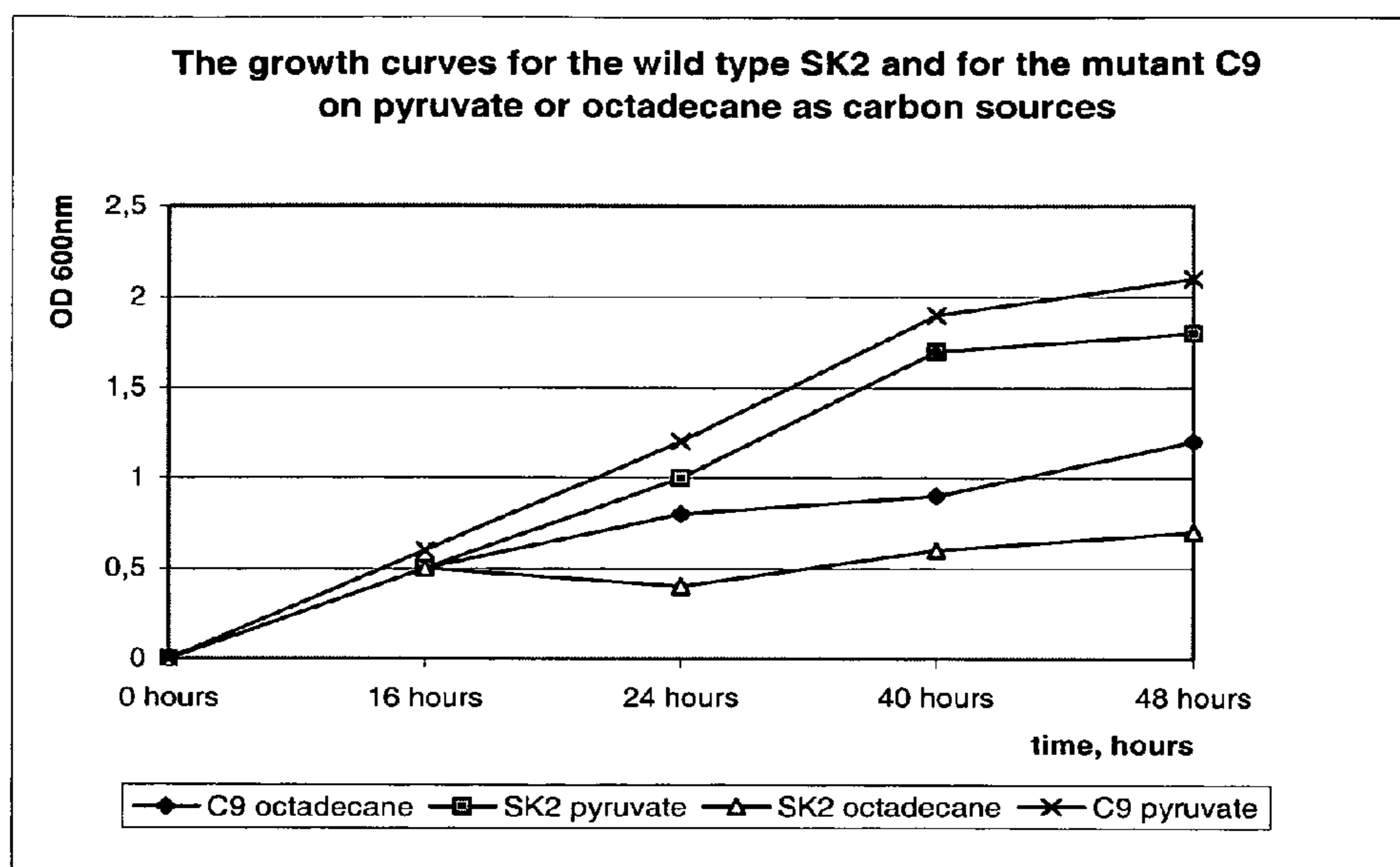


Figure 4

Table 1. *In silico* analysis of sequence data on the genes for polyhydroxyalkanoate production and mobilization in *Alcanivorax borkumensis* SK2

Gene number in <i>Alcanivorax</i>	Annotated as	Homologous to	Closest homolog % similarity, identity	Function
Abo_1335	phaC synthase	PhaC synthase	<i>Acinetobacter</i> sp. ADP 1 30% identity, 53% similarity	PHA synthesis, last step
Abo_2062	phaC synthase	PhaC synthase	<i>Acinetobacter</i> sp. ADP 1 34% identity, 56% similarity	PHA synthesis, last step
Abo_1526	dienelactone hydrolase family protein	poly(3-hydroxyalkanoate) synthase	<i>Thiocystis violacea</i> 29% identity 45% similarity	PHA synthesis, last step
Abo_2240	Conserved hypothetical protein	poly(3-hydroxybutyrate) depolymerase	<i>Alcaligenes faecalis</i> 29% identity 41% similarity	PHA depolymerization
Abo_1424	Conserved hypothetical protein	D(-)-3-hydroxybutyrate oligomer hydrolase	<i>Ralstonia pickettii</i> 24% identity 46% similarity	Intracellular PHA depolymerization
Abo_1041	transglutaminase-like superfamily domain protein	polyhydroxyalkanoate depolymerase precursor	<i>Rhodopirellula baltica</i> SH 1 36% identity 59% similarity	PHA depolymerization
Abo_915	Conserved hypothetical protein	PhaF, poly(hydroxyalkanoate) granule associated protein	<i>Acinetobacter</i> ADP1 sp. 37% identity 60% similarity	Granule formation, regulation of PHA synthesis
Abo_425	Hypotetical protein	PhaF, polyhydroxyalkanoate synthesis protein	<i>Pseudomonas aeruginosa</i> PAO1 26% identity; 52% similarity	Granule formation, regulation of PHA synthesis

Figure 5

Table 2. Polyhydroxyalkanoate composition in cells of *Alcanivorax borkumensis*

Sample	Amount PHAs (mg/l)	PHA monomer composition of hydroxyalkanoates (mol%)			
		(C6)	(C8)	(C10)	(C12)
SK2 pyruvate	6.5	2	24	46	28
SK2 cells octadecane	18	2	20	48	30
C9 mutant cells octadecane	n.d.	n.d.	n.d.	n.d.	n.d.
C9 mutant supernatant octadecane	2960	9	15	35	41
C9 mutant cells pyruvate	n.d.	n.d.	n.d.	n.d.	n.d.
C9 mutant supernatant pyruvate	134	4	18	37	39

n.d. not determined. The amount of PHA was too low for quantification.

Results indicate the average of two independent analyses. The standard deviation did not exceed 5%.

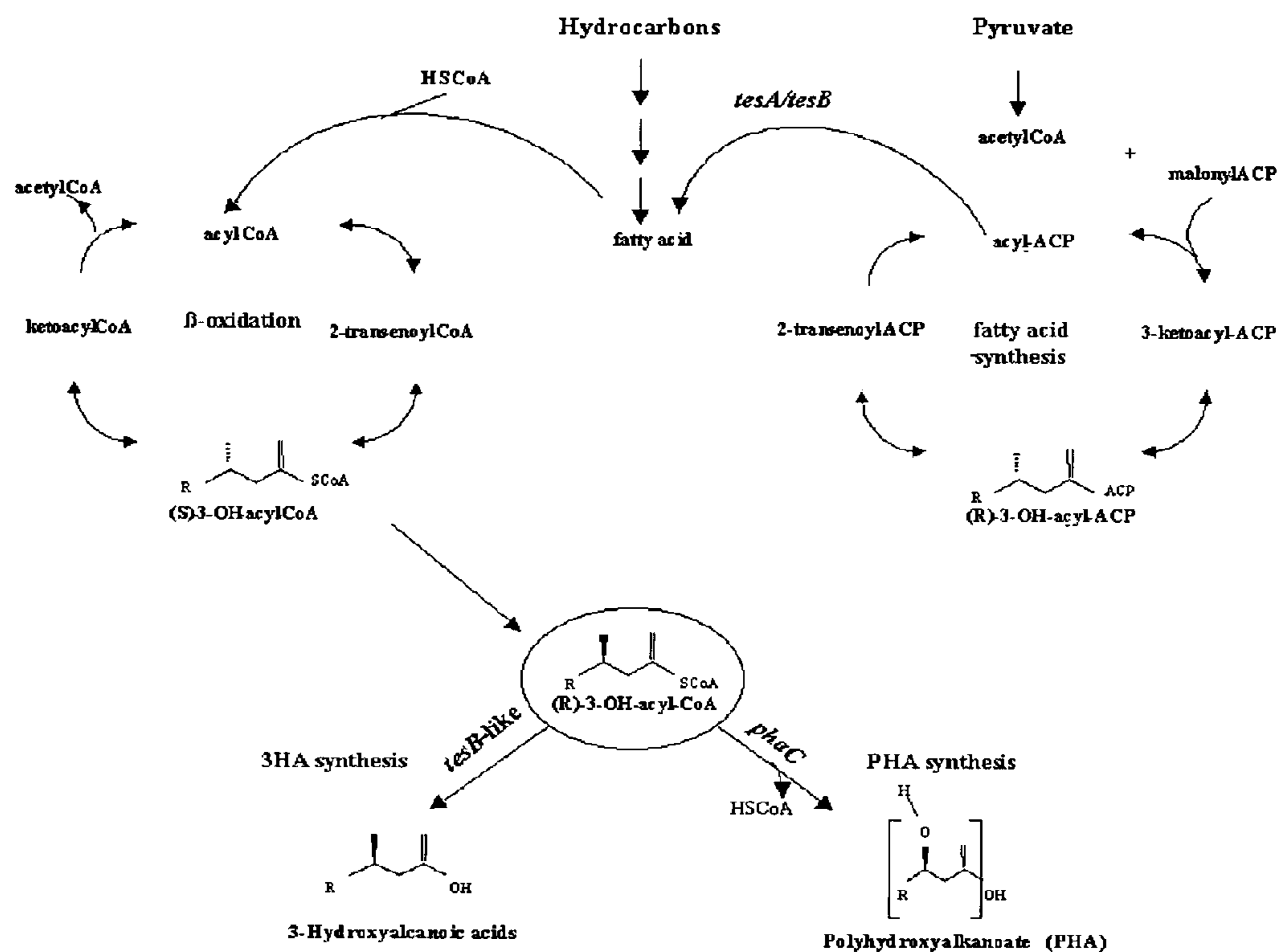
Figure 6

Table 3. Molecular weight of PHA from *A. borkumensis*

Sample ¹	MW PHA (kDa)			
	<i>Hydroxyhexanoate</i> (MW monomer: 132.2 Da)	<i>Hydroxyoctanoate</i> (MW monomer: 160.2 Da)	<i>Hydroxydecanoate</i> (MW monomer: 188.3 Da)	<i>Hydroxydodecanoate</i> (MW monomer: 216.3 Da)
C9 Mutant octadecane	330	325	207	540
C9 Mutant pyruvate	316	327	217	549
Wild type octadecane	250	247	180	432
Wild type pyruvate	258	241	189	426

¹Supernatants were used when analyzing C9 mutant, and cells when using wild type strains.

Figure 7



Hypothetical pathway of *A. borkumensis* SK2 grown hydrocarbons/pyruvate

Figure 8

The operon structure

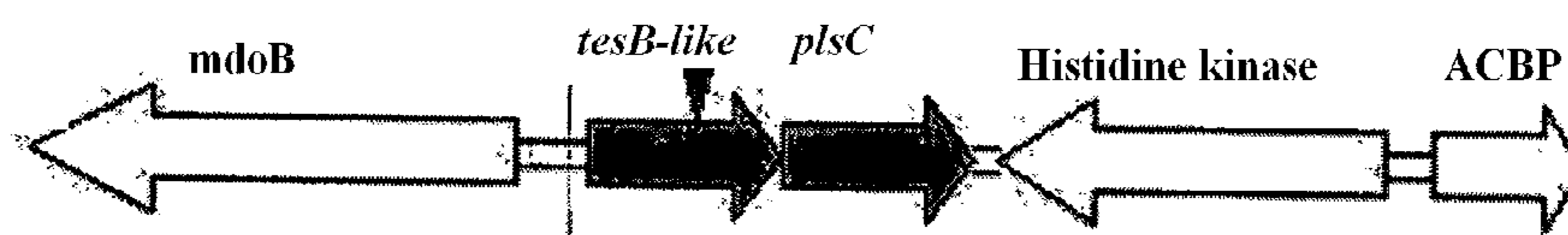
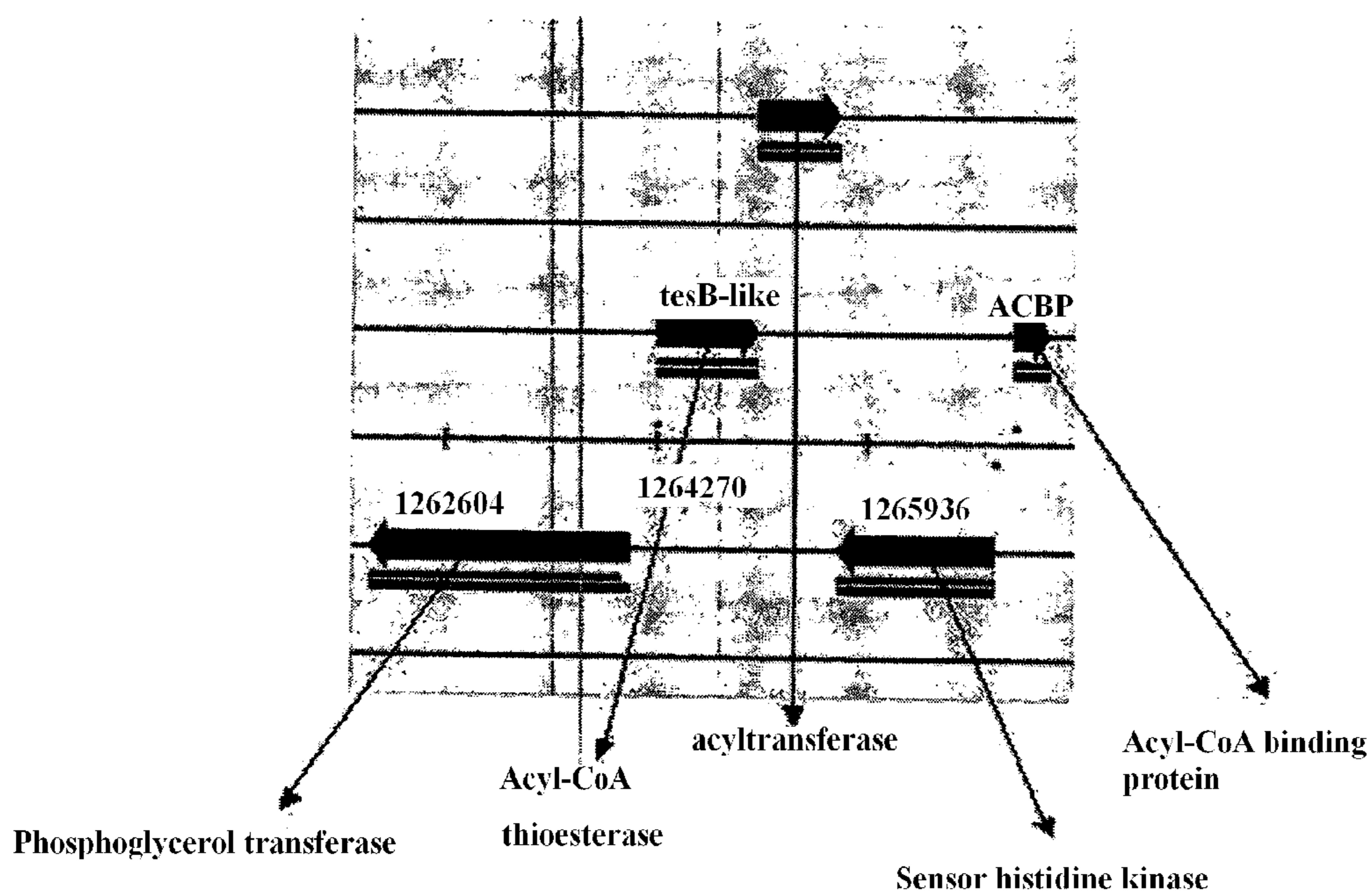


Figure 9

Table 4. Putative Acyl-CoA thioesterase proteins in other gram-negative bacteria encoded by genes homologous of the tesB-like gene of *A. borkumensis* SK2 (Abo_1044)

Species	Name of the homologous gene	% Identity (% similarity) to Abo_1044
<i>Pseudomonas putida</i> KT2440	putative acyl-CoA thioesterase II	40 (60)
<i>Pseudomonas aeruginosa</i> PA01	hypothetical protein PA2871	41 (56)
<i>Pseudomonas syringae</i> pv B728a	acyl-CoA thioesterase II, putative	42 (59)
<i>Pseudomonas fluorescens</i> PfO-1	Acyl-CoA thioesterase	42 (59)
<i>Idiomarina loihiensis</i> L2TR	tesB-like acyl-CoA thioesterase	42 (57)
<i>Acinetobacter sp.</i> ADP1	putative acyl-CoA thioesterase II	36 (54)
<i>Caulobacter crescentus</i> CB15	hypothetical protein CC2472	29 (47)

Figure 10

Nucleic sequences of the two genes *tesB-like* (underlined) and „putative acetyltransferase“ (black colour) consisting an operon. The first line is the region upstream from the *tesB-like* gene. The start codon of the *tesB-like* gene is atg and marked in bold letters. The Tn5 insertion took place at position 527 within *tesB-like* gene and marked with //. Downstream from the *tesB-like* gene is „putative acetyltransferase“ gene. There is overlap in 3 bp.

```
ggcttcgcag gaatggtaaa taaagcaccg caaattcaaa acacgcagaa gtgagagacc
atgacattcg atgagattct agccacgatt gatggccagg gtaacgccac gtttccagaa
gggtgggggc agggccggac cttatattggt ggcttggtgg gggcgggtgct gtttgaacat
ttagaaaaaa ccgtggctcg cgggagggtt ctacgtagtt tttctctctc ttttgtegcc
cctgcgggtgc cgggtccggt ggcactagac gagactgtgt ttcgggaagg caaatccggt
atgcaggcca tgggtctcgc ccgtcaagga gggcagggtg tggcggttat gttggccagc
tttggggcca gtcgccaatc cagtgtagtg gtagaagggc catcggcccc agtgatgaaa
tctccagagc agagtatttc ggtaccgttt atcaaaggat tgacgcggga ttttttttcc
cattttaata tccattacgc agaaggcatg ccgcccgttca gtggcagttc tgagcccgat
tacgggtggtt acatgggggtt tactgtgccc ccggaaacca tgagcac//cgc agccctgatt
gcattggtgg atacctgggc gccttcagtt ttacctctgc tcaaagggcc agcaccgcc
agttccttga cctggaccat ggagctcttg gatgacccta gtgtccactc gccggagact
ttatggcaat atcgggtaaa cacggacca tgcagtgatg gctatggtea aagtcaggcg
gtggtttggg atgctgcccgg taaggetgtg gcgcttgagtc gacaaacctt tacggtattt
gcatgaaatc ggaactggtg ccactcacgg ccgaggacttt aggtaagcaa gtgcctcgtc
gtgggcaactg gctggtggcg gcgctagggc ggctgatttt gactgttatg gggtggcgta
ttgttggtga tttgcccgat acgcctcggg cagttttggc ggtggcgcca cacacgtcga
acatagacgg ggtcattggt atcagtgcta ttcagttttt gcgcttgat gtgcgcttca
tgggtaagca cacgttgttt aaaggctcgtc ttgggcccgtt catgtactgg ctgggcccga
tcctgtgaa tcgagaaagt gccagggatg tgggtggacca gacgacgtcg gtgatggggg
aacaccatt ttggcttggg ctaacgccgg aggggacgcg taaaggccc aagcgttggg
aaaccgggtt ttaccgtatt gctgagcaaa tgcagggtgc gattgtcgtg ttaggtttct
gttaccggcg ccggcaggtc cggattgtag attgctttct gccgacgggc gatattgatg
ctgatatggc gcgaatgacc gcgtcgttgg cggatattgt tccgcgcaaa cctgcgcagt
tatccgcccc gctgaaagcg gaaaaagctg ctccgcccgt tgattgatc agacgttggc
agttttgccg gactgatcaa ggagatgctg gcggtgctgg gcggat
```

Figure 11

start and stop codons are marked in bold. The Tn5 insertion took place at position 557 and is marked //.

atgacattcg atgagattct agccacgatt gatggccagg gtaacgccac gtttccagaa
gggtgggggc agggccggac cttatttggg ggccctgggg gggcgggtgc gtttgaacat
ttagaaaaaa ccgtggctcg cgggagggtt ctacgtagtt tttctctctc ttttgtegcc
cctgcggtgc cgggtccggt ggcactagac gagactgtgt ttcgggaagg caaatccgtt
atgcaggcca tggctctcgc ccgtcaagga gggcagggtg tggcggttat gttggccagc
tttggggcca gtcgccaatc cagtgtagtg gtagaagggc catcgccccc agtgatgaaa
tctccagagc agagtatttc ggtaccgttt atcaaaggat tgacgccgga tttttttcc
cattttaata tccattacgc agaaggcatg ccgccgttca gtggcagttc tgagcccgat
tacggtggtt acatgggggt tactgtgccg ccggaaacca tgagcaccgc agcgtgatt
gcattggtgg atacctg//ggc gccttcagtt ttacctctgc tcaaagggcc agcaccgcc
agttccttga cctggaccat ggagctcttg gatgacccta gtgtccactc gccggagact
ttatggcaat atcgggtaaa cacggaccaa tgcagtgatg gctatggtea aagtcaggcg
gtggtttggg atgctgcggg taaggctgtg gcggtgagtc gacaaacctt tacggtattt
gcatga

Figure 12

Amino acid sequence of the tesB-like acyl-CoA thioesterase

MTFDEILATI DGQGNATFPE GWGQGRTLFG GLVGAVLFEH LEKTVARGRF LRSFSLSFVA
PAVPGPVALD ETVFREGKSV MQAMVSARQG GQVVAVMLAS FGASRQSSVV VEGPSAPVMK
SPEQSISVPP IKGLTPDFFS HFNIHYAEGM PPFSGSSEPD YGGYMGFTVP PETMSTAALI
ALVDTWAPSV LPLLKGPAPA SSLTWTMELL DDPSVHSPET LWQYRVNTDQ CSDGYGQSQA
VWDAAGKAV ALSRQTFTVF A

Figure 13

Nucleotide sequence of miniTn5 Km coding repeats functional in transposition and neomycin phosphotransferase, responsible for neomycin and kanamycin resistance. The repeats functional in transposition are marked in bold. The first repeat (1.....19) is called Tn5 I end, the second repeat (2338...2356) is called Tn5 O end. In the case of Tn5 Str/Sp element the kanamycin resistance gene is replaced by streptomycin resistance gene.

CTGTCTCTTGATCAGATCTGGCCACCTAGGCCGAATCCCGGGGATCCGGTGATTGATTGAGCAAGCTTT
 ATGCTTGTAACCGTTTTGTGAAAAAATTTTAAAATAAAAAAGGGGACCTCTAGGGTCCCCAATTAATT
 AGTAATATAATCTATTAAAGGTCATTCAAAGGTCATCCACCGGATCACCTTACCAAGCCCTCGCTAGAT
 TGTTAATGCGGATGTTGCGATTACTTCGCCCAACTATTGCGATAACAAGAAAAGCGCCTTTCATGATATA
 TC'TCCCAATTTTGTGTAGGGCTTATTATGCACGCTTAAAAATAATAAAAAGCGACTTGACCTGATAGTTTG
 GCTGTGAGCAATTATGTGCTTAGTGCATCTAACGCTTGAGTTAACCGCGCCGCGAAGCGGCGTCGGCTTG
 AACGAATTGTTAGACATTATTTGCCGACTACCTTGGTGATTCGCCTTTCACGTAGTGGACAAAATCAACC
 AACTGATCTGCGCGAGCTTCACGCTGCCGCAAGCATCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACAC
 GTAGAAAGCCAGTCCGCAGAAACGGTGCTACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGG
 AAAACGCAAGCGCAAAGAGAAAGAGGTAGCTTGCAGTGGGCTTACATGACGATAGCTAGACTGGGCGGTT
 TTATGGACAGCAAGCGAACCAGGAAATGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAG
 TAAACTGGATGGCTTCTTGGCCGAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGG
 ATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGG
 CTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGC
 AGGGGCGCCCGGTTCTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGC
 GCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGA
 AGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGA
 AAGTATCCATCATGGCTGATGCAATGCGGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCA
 CCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTG
 GACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCCGCGAGGCTCAAGGCGCGCATGCCCGACGGCG
 AGGATCTCGTTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGG
 ATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATT
 GCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGC
 AGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGAC
 CAAGCGACGCCAACC'TGCCATCACGAGATTTTCGATTCACCGCCGCCCTTCTATGAAAGGTTGGGCTTCG
 GAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCA
 CCCC'GGGCTCGATCCCC'TCGCGAGTTGGTTCAGCTGCTGCCTGAGGCTGGACGACCTCGCGGAGTTCTAC
 CGGCAGTGCAAATCCGTCGGCATCCAGGAAACCAGCAGCGGCTATCCGCGCATCCATGCCCCGAACTGC
 AGGAGTGGGGAGGCACGATGGCCGCTTTGGTCGACCCGGACGGGACGGATCAGTGAGGGTTTGCAACTGT
 GGGTCAAGGATCTGGATTTTCGATCACGGCACGATCATCGTCGGGAGGGCAAGGGCTCCAAGGATCGGGCC
 TTGATGTTACCGAGAGCTTGGTACCCAGTCTGTGTGAGCAGGGGAATTGATCCGGTGGATGACCTTTTGA
 ATGACCTTTAATAGATTATATTACTAATTAATTGGGGACCC'TAGAGGTCCCTTTTATTTTAAAAATT
 TTTTCAAAAACGGTTTACAAGCATAAAGCTTGCTCAATCAATCACCGGATCCCCGGGAATTCGTCGACA
 AGCTGCGGCCCGCTAGGCCGTGGCCGA**ACTTGTGTATAAGAGTCAG**

Figure 14

Amino acid sequence of neomycin phosphotransferase responsible for neomycin and kanamycin resistance.

MIEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLVFKTDLSGA
LNELQDEAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLSSHLAPA
VSIADAMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPA
ELFARLKARMPDGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALAT
RDIAEELGGEWADRFLVLYGIAAPDSQRIAFYRLLDEFF

Figure 15

Nucleotide sequence of a putative acyltransferase (Abo_1045), start and stop codons are marked in bold. There is a 4 nucleotides overlap with the previous open reading frame of tesB-like acyl-CoA thioesterase (Abo_1044)

```
atgaaatcgg aactggtgcc actcacggcg cggacttttag gtaagcaagt gcctcgtcgt  
gggcactggc tgttggcggc gctagggcgg ctgattttga ctggtatggg gtggcgtatt  
gttgggtgatt tgcccgatac gcctcgggca gttttggcgg tggcgccaca cacgtcgaac  
atagacgggg tcattggtat cagtgcatt cagtctttgc gcttggatgt gcgcttcatt  
ggtaagcaca cgttgtttaa aggtcgtctt gggcggttca tgtactggct gggcggcatc  
cctgtgaatc gagaaagtgc cagggatgtg gtggaccaga cgacgtcggg gatgggggaa  
acaccatttt ggcttgggct aacgccggag gggacgcgta aaggcgccaa gcgttggaaa  
accgggtttt accgtattgc tgagcaaatg caggtgccga ttgtcgtggt aggtttctgt  
taccggcgcc ggcaggtccg gattgtagat tgctttctgc cgacgggcca tattgatgct  
gatatggcgc gaatgaccgc gtcgttggcg gatattgttc cgcgcaaacc tgcgcagtta  
tccgccccgc tgaagcgga aaaagctgct cgcggcattg attga
```

Figure 16

Amino acid sequence of “putative acyltransferase”.

MKSELVPLTA RTLGKQVPRR GHWLLAALGR LILTVMGWRI VGDLPDTPRA VLAVAPHTSN
IDGVIGISAI QSLRLDVRFM GKHTLFKGR LGRFMYWLGGI PVNRESARDV VDQTT SVMGE
TPFWLGLTPE GTRKGAKRWK TGFYRIAEQM QVPIVVLGFC YRRRQVRIVD CFLPTGDIDA
DMARMTASLA DIVPRKPAQL SAPLKAEKAA RGID

Figure 17

**Nucleotide sequence of putative acyl-CoA thioesterase II of *Pseudomonas putida* KT2440
(GeneID:1045294)**

ATGACTTTCAACCAACTGCTCGACGCCGTGCGGGCCAACCCGGATTCCGTCAGCATTCCGCCAGCTGGG
CCCAGGGGCGCGCTGCCTTTGGCGGGCTGATGGCGGCCATGGTCTATGAAACCATGCGCCTCAAGATCAG
CGACAACCGCCCGGTACGCTCATTGGCCATCAGCTTCGTGGCACCCGCGGGCGGGATGTGCCATCCGC
TTCGAGGTGGAGGTTTTCGCGAAGGCAAGGCGGTTAGCACGTTGCTGGGCCGCGCTGTTTCAGGATGGCC
AGGTGGTGACTTTGGTGCAGGGCAATTCGGTGCGGGCGCCCTTCGGTGGTTCGAAGTGCCGGCGTTGCC
GGCCATCGAAATGCCTGCGCTCGATGAGGCGGCCCGGAGTTGCCCTATATCAAAGGCGTTACCCCTGAG
TTCATGCGGCACGTGGCCCTGCGCTGGGCAGTAGGTGGGCTGCCGTTTCAGTGGCAATCAGTCGCGCAAGA
TGGGCGGCTGGGTGCGCTTTCGGGATGTGGTGGGAAGAACAGGTGAACGAGGCGCACCTGTTGGCGTTGGT
CGATGCCTGGCCGCCAGCCTCATGCCGTTTCTCAAGCAGCCCGCTGCGGGCAGTACGTTGACCTGGACC
ATCGAGTTCATCCAGCCAACGGCGAAGCTGTCGACCCTGGATTGGTGCCGCTACTGTGTGGAGACCGAGC
ATGCGCGGGATGGCTATCGGCATGCTGCTCGGCCGTTGTGGACGGCGCAGGGCGAGTTGTTGGCGTTGAG
CCGGCAGACCGTCACCGTTTTTCGCCTGA

Figure 18

**Nucleotide sequence of hypothetical protein PA2871 of *Pseudomonas aeruginosa* PA01
(GeneID:882599)**

ATGAATTTTTCCGAATTGATCCAGGCGGTCCGCCGCGACCCTTCCTCGGTGGTAGTACCGGCCAGTTGGG
GCCAGGGCCGCGCCACCTTCGGTGGCCTGGTGGTGGCGTTGGCCTACGAGGCCATGCTTGCGGTGGTCGA
GGCGGGGCGTCCGTTGCGCTCCATCGGCGTCAGCTTCGTCGGACCGCTGGCCCCGAGCAGCCGGCGAGC
TTCAGCGCCCGGTTGTTGCGCGAGGGCAAGGCGGTGAGCCAGGTCCAGGTGAGGTCCGTCAGGGCGAGC
AGGTGGTGACGCTGGTCCAGGCCAGTTTCGGCGTCGCCCGCGCATCGGCGGTGGCGGTGGAAGCGTTGCC
GGCGGCCGGGATGAAGGGCCCCGAAGAGAGCCAGGAGCTGCCCTATATCCGTAACGTGACCCCGGAGTTC
ACTCGCTACATCGCCATGCGCTGGGCAGTGGGCGGCCTGCCGTTCTCTTCGAACAAGTCGCGCCAGATGG
GCGGCTGGATGCGTTTCCGCGACGAACCCGAGGGAGAGCCATGGAGGTTTCCCACCTGCTGGCGCTGCT
CGACTCCTGGCCGCCGGCGCTGTTGCCGCACCTGGGCACCCCGGCGATGGCCAGCTCGCTGACCTGGACC
GCCGAGTTCCTCCAGCCGCTGCCGCAGCAAGGCAGCGGCGACTGGTGCCGTTACCTGGCGGAGATCGAGG
AGGCGCGTGACGGCTACGGCCACGTGGCGGCGCGGATGTGGAGCGCCGACGGCCAGTTGCTGGCGATCAG
CCGGCAGATGGTCACGGTGTTCGGCTGA

Figure 19

Nucleotide sequence of acyl-CoA thioesterase II, putative of *Pseudomonas syringae* pv B728a (GeneID:3367571)

ATGACCTTTTCTGAACTGATCGATGCGCTGCGTCGCGATCCGCGCTCTGTCACGATCCCTGCCGAATGGT
CTCAAGGGCGTGCCTGCTTCGGCGGGCTGATGGCTGCGCTGACCTACGAAGCGATGCGTGCACAGGTGCC
GGAAGGGCGGCCGGTTCGTTTCGTTGGCGATCACCTTTGTCGGGCCGGCCGCGCCCCGGTGTGCCGATTGCT
TTCGAGGTCGACACCCCTGCGCCATGGCAAGGCAGTCAGTCAGGTGCTGGGACGCGCCATGCAGAACGGTC
AGGTCATGACCCTGATACAAGGCAGCTTCGGTGCCCCCTCGGGAATCGATGATCACCGTTGCCGCAGAGGC
CGCGCCGGTCCTTAAACCGGTTGATCAATGTCCGGAGCTGCCGTTCCGCCAGCGGCGTGATGCCTGATTAC
CTGCGCTTCATGGACATCCGCTGGGCGTTGGGCGGCATGCCATTCAGTAATACCCGATCACCGGCGATTG
GCGGCTACGTGCGCTTTCGCGATACGCCGCACGCCACGCCCATGAGCGAAGCGCACATTCTGGCGCTGGT
GGACACCTGGCCGCTGCGGTACTGCCGCACCTGGACAAACCGGCCCCCGGCAGCTCACTGACCTGGACC
ATTGAGTTCGTCCAGCCCCAGCCGTCGCTCGATACCCTGCAGTGGTGCAGCTACCGCAGTCATCGAGC
ATGCCCGCGATGGCTATGGCCATACCGCCGCGGCATTGTGGAGCCCCGACGGCGAGCTGATCGCAATCAG
CCGCCAGACGGTTACCGTATTTGGCTGA

Figure 20

**Nucleotide sequence of acyl-CoA thioesterase of *Pseudomonas fluorescens* PfO-1
(locus_tag=Pflu02003109)**

ATGCGCTTTTGGCGATCTGATCGATGCTGTCCGTCGTC A ACCGGAGGTCACGATTCCGGCCGGAGTGGGGCC
AGGGCCGGGCCAGTTTTGGCGGGCTGGTGGCCGCGCTGCAATTTGAAGTGATGCGCACCAAGGTTCCGAC
CGATCGGCCGGTACGTTTCGCTGGCGATCACCTTCGTCGGCCCGGTCGAGCCCGAAGTGCCGGTGAGTTTT
GAAGTCGAGGTACTACGCGAAGGCAAAGCGGT CAGCCAGGTGCTGGGGCGTGCTGTGCAGAACGGTCAGG
TGGTGACGATGGTGCAAGGCAGCTTCGGGGCTTCGCGGCCATCGGAAGTGGCGGTTGAAGCCTATCCCGC
GCCGAAATGAAGCACTGGGACGATTGCCAGGAACTGCCGTACATCAAAGGCGTAACCCCGAGTTCATG
CGTCATCTGGCGATGCGCTGGAGCGTTGGCGGGATGCCGTTACCGGCAATCAATCGCGGCTGATGGGTG
GCTGGGTGCGCTGCGTGGGGATGTGAAGGAAGAGTCGGTCAACGAAGCGCACCTGCTGGCGCTGGTCTGA
TGCCTGGCCACCAGCGCTGTTGCCGTACCTGAAGAAACCGGCACCGGGCAGTACGCTGACCTGGACCATC
GAATTCGTTACGCCGTTACGCGATTTGAGTACGCTGGATTTTGGCCAATACCTGGCGGACATCGAGTATG
CCGCCGACGGTTACGGCCACGTCGCCGCCAAGCTGTGGAGTGC GAAGGGTGAAGTGAATGTCATGAGTCCG
GCAGACGGTGACGATCTTCGCCTGA

Figure 21

**Nucleotide sequence of tesB-like acyl-CoA thioesterase of *Idiomarina loihiensis* L2TR
(locus_tag='IL0656')**

ATGAACTTCCACACAGCTGTAGAACAAATCGTTGCGGATAAGAACAATCAAGTCATTGAACTACCTTCCG
GCTGGGCTCAGGGACGCGCGTTTTTCGGCGGATTCAGCGGAGCATTGGCTGCTCAGTTTTTGTGAAACA
ATTTCCGATTGAATATCATCTTCGTTCCATGAGTATCTCTTTGTGTCGCGCCTGCTGAACCGGGTGAGGCT
GAGTTAAATTACCGAATTTTGC GCGAGGGAAAATCGGTTATTCAGGTTGCTGTGCGAACTGCAGCAGCAAG
GGCAGATTATGTTGTCCTGTCCTGGCGAGCCTGGGCAAAGGCCGAAGTTCAACGGTTACAGTGGTAAGTGA
AACGCCACCCGATCTTAAAACCATCAACGACGGACCAGGTTTACCTGAAGCGGATATTGTCCCAGAGTTT
GCGAAAAACTTCGATTACCGTATTACGTCAGGTGGTATGCCATTTAGCGGACAACCGGGAAGAACTTTTG
GTGGCTGGATACGTTTTTCGTGAAGAACAACAGCCGCTGACAACGGCAACTATACTGGCTTTAGTTGACGC
CTGGCCACCGGCAGTCTTGCCATCTGGACAGCCCTGCGCCGGCCTCGTCTTTAACCTGGACTATTGAG
TTTCCCGATATTCCTCTACAAAGTTTCAGTAGCCACGACTGGTTTTAGTACGAAGCTTTTATTGAGCATG
CTGAAAATGGTTATGGGCACAGCCGCGGGTCTGTGGAGTGAGAAGGGCGAGTTATTAGCTATAAGTCG
ACAGACTTTTACGGTATTTGCGTAA

Figure 22

**Nucleotide sequence of putative acyl-CoA thioesterase II of *Acinetobacter* sp. ADP1
(locus_tag="ACIAD0341)**

ATGAGTGATGCTATGCCTTTAGATCAGACATTGGAACAAC TGACGCAAGATGAATGGATTGATATTCCCC
AAGGCTGGTCGCAGGGAAGAACGATTTATGGTGGATTGGTTGCAGGCTTGCTCATGCATAAAGCATTAAG
TGTGATGAATGATGAGTCCAAAAATCTGTTAAGTACCAGCATTACTTTTGTGGGACCAGTGAATGAAGGG
CGGGTACGACTAACAGTTGAAATCTTGCGGCAGGGTAAATCTGTCACCACAATTGAGGCACGTTTATGGC
AGGACCAGGCTGTTCAAACCATCTTGATCGCAAGTTTCGGACAGCCGCGTTCCTTGAAATTTTGTGCT
TAACTTACCCGAGGCACCAGACTATTTGTCTCCCGAGCAGTTC CAAGAATGCCTTTTGTA AAAATGATG
CCAGAATGCTATCAACAATTCGATCTGCGATGGGCAGAAGGTCATTATCCCATGACTCAACAAGCTCCAG
ATTTTGGTGGCTGGTGCCGTTACGATATCCAAAAACATTCACCCGCGAGCATTGAATGTGGCTGATTTGTT
AATTTTGATGGATATCTGGCCACCAGGGGFACTGCCCATGTTTCAAACCATCGCACCTGCCAGTTCTCTA
ACATGGCATCTCACTTTTGTTCGTCGCCGTTGCTTATGAGTTACATGATTGGTTTAAGTATCAGGTCGTCA
CGCAGCATGCTGCCTTTGGCTATGCTACAGAATATGCGCATTATGGGATGCTCAA AACCGTCTGATTGC
TATTTTCGAGGCAGACAGTTACTGTTTTTGCCTAG

Figure 23

(GeneID:943632)

ATGACGCTCTACACCGACCTCGTCGCGGCCATCGCCTCGACCGAAACTGGCTTTTCCGCCCATGTCTCCG
ACGACTGGAAGCAAGGCCGCACCACCTATGGCGGCTTGAGCGGCGCCTTGTGCGTCGAGGCCGCCCTGCG
AGCCTTTCCCGAGGCCCTCCTCTGCGCTCGGCGCAATTCGCCTTTGTGCGGCCCGGCGGCCGGCGAGTTG
GCGATCTCGGTGCGGCCGCTGCGGCAGGGCAAGTCGACGCTTTTCGTCGCCGTCGATCTGATCGGCCAAC
AGGGCGTGGCCACCCACGGCGTGTGACCTTCGGCGCGGCGCGGACCTCAGCCATCTCGTACGAAGAAGT
CCTCTGCCCGCCCCTGGCGCCGGCCGGCGCCTGTGAGCTGTTCTTCCCCGAAAATCGCCAGGGCGCGCCG
CACTTCTCGGCGCAGTTCGAGGTGCGCAAGGCCGGCGGCACGCGCCCCCTGGCCGGGGGTGAGCCGGAAT
ATCTGCTGTGGATCCGCCACCGCGATCCGGCCGCGACCTCGATCTCGGCCCTGGTGGCGTTGGCCGACAT
GCCGCCGCCCCCGGCCATGGCGCTGTTCCCGCAGTTTGGGCCGATCTCGACCATGACCTGGTCGCTGGAT
ATCGTGGGCCTGCCCGAGGCGGACGACGACGGCTGGCGGCTGCTGCGCACCCGGGCCGAGACCATCGGCCG
ACGGCTACTCGACCCAGGAGATGCATCTGTGGGACGCCAAGGGCCGCCCGCTGGTCCTGGCGCGACAGAA
CGTGGCGATCTTCGTCTGA

Figure 24

**Nucleotide sequence of hypothetical protein ELI0992 of *Erythrobacter litoralis*
HTCC2594
(locus_tag=ELI0992)**

ATGTCCGTTTCCGATCTTCGTGCGCCGATCACGTCCGAAGGTGGCGCTGTCACTCTTCCAGCCGACAAAT
GGCTGCAGGGCCGCACGCTCTTTGGCGGAGCCTCGGCGCTTGTCGCCTACACCGCCGCGGTGCGCGCTTT
CCCCGATCTCCCGCCCTTGC GCGCAGCGCAGATCGGATTTGTCGCGCCGGTCGGAAAGGACGTGGAGACG
CGGGCCGCAATGGTCCGACAGGGTCGCAACGTCGCGCAGGTGCGCAGCGAACTGCTGGTTCGAAGGCAAGG
TCGCGCTCACCGCATTCTGGCTGTTTCGGAACCGGTCGCGAGGCCAACGCCGTACATGCCGCTGCCAAGGC
CGATCCCTGGCCCGGCGCACCGGAAGAGAACGATTCCGCGATGACCGACAAGGGCCCGCCTTTCATCGTC
AACAATTTGACATTCGCCGCGCGCAGGAAACGCAAGGCCCGGCGAACCGATCGTCCGGCGCTGGTTCA
GGCTGACCGATCGGGGCGAGCTCGATCGCGTATCGGAGCTGATCCTGGTGGGCGATACGCTGCCCCGGG
CGCCATGCGCGCGATGCAGCGCCAGGGCCCGATCAGCTCGATCAACTGGTTCGTTCAATATTCGATGCG
GAACTCGGCACGCGCGACGGCTGGTGGCTCGGCGAGACCGCCAGCCAGCATGCCGGTGCAGGCTATTCGA
GCGAGCGGCTACGGCTCTGGAATGCCGACGGCGTGCAGGTGATGGACGGATTGCAATCCGTTGCCGTCTT
CGGCTGA

Figure 25

**Amino acid sequence of putative acyl-CoA thioesterase II of *Pseudomonas putida* KT2440
(protein_id="NP_744457.1)**

MTFNQLLDAVRANPDSVSI PPSWAQGRAAFGGLMAAMVYETMRLKISDNRPVRS LAISFVAPAAADVPIRFEVEV
LREGKAVSTLLGRAVQDGQV VTLVQGNFGAGRPSVVEVPALPAIEMPALDEAAPELPYIKGVTPEFMRHVALRWA
VGGLPFSGNQSRKMGGWVRLR DVVVEEQVNEAHL LALVDAWPPSLMPFLKQPAAGSTLTWTIEFIQPTAKLSTLDW
CRYCVETE HARDGYGHAAAALWTAQGELLALS RQTVTVFA

Figure 26

**Amino acid sequence of hypothetical protein PA2871 of *Pseudomonas aeruginosa* PA01
(protein_id="NP_251561.1)**

MNFSELIQAVRRDPSSVVVPASWGQGRATFGGLVVALAYEAMLAVVEAGRPLRSIGVSEFVGPLAPEQPASFSARL
LREGKAVSQVQVEVRQGEQVVTLVQASFGVARASAVAVEALPAAGMKGPEESQELPYIRNVTPEFTRYIAMRWAV
GGLPFSSNKSRQMGGWMRFRDEPEGEPMEVSHLLALLDSWPPALLPHLGTAMASSLTWTAEFLQPLPQQSGDW
CRYLAEIEEARDGYGHVAARMWSADGQLLAISRQMVTVFG

Figure 27

Amino acid sequence of acyl-CoA thioesterase II, putative of *Pseudomonas syringae* pv B728a (protein_id="YP_235138.1)

MTFSELIDALRRDPRSVTI PAEWSQGRACFGGLMAALTYEAMRAQVPEGRPVRS LAITFVGPAAPGVPIA FEVDT
LRHGKAVSQVLGRAMQNGQVMTLIQGSFGAPRESMITVAEEAAPVLKPVDQCPEL PFASGVMPDYLRFM DIRWAL
GGMPFSNTRSPAIGGYVRF RDTPHATPMSEAHILALVDTWPPAVLPHLDKPAPGSSLTWTIEFVQPQP SLDTLQW
CSYRAVIEHARDGYGH TAAALWSPDGELIAISRQTVTVFG

Figure 28

**Amino acid sequence of acyl-CoA thioesterase of *Pseudomonas fluorescens* PfO-1
(protein_id="ZP_00264181.1)**

MRFCDLIDAVRRQPEVTI PAEWGQGRASFGGLVAALQFEVMRTKVPTDRPVRSLAITFVGPVEPEVPVSF
EVEVLREGKAVSQVLGRAVQNGQVVTMVQGSFGASRPSEVAVEAYPAPEMKHWDDCQELPYIKGVTPEFM
RHLAMRWSVGGMPFTGNQSRMGGWVRLRGDVKEESVNEAHLALVDAWPPALLPYLKKPAPGSTLTWTI
EFVQPLRDLSTLDFCQYLADIEYAADGYGHVAAKLWSAKGELIAMSRTVTIFA

Figure 29

**Amino acid sequence of tesB-like acyl-CoA thioesterase of *Idiomarina loihiensis* L2TR
(protein_id=AAV81497.1)**

MNFHTAVEQIVADKNNQVIELPSGWAQGRAFFGGFSGALAAQFLKQFPIEYHLRSMSISFVAPAEPGEAELNYR
ILREGKSVIQVAVELQQQGQIMLSCLASLGKGRSSTVTVVSETPPDLKTINDGPGLPEADIVPEFAKNFDYRITS
GGMPFSGQPGRTFGGWIRFREEQQPLTTATILALVDWPPAVLPHLDS PAPASSLTWTFIEFPDIPLQSFSSHDWF
QYEA FIEHAENGYGHSRAGLWSEK GELLAISRQTFTVFA

Figure 30

**Amino acid sequence of putative acyl-CoA thioesterase II of *Acinetobacter sp.* ADP1
(protein_id=CAG67294.1)**

MSDAMPLDQTLLEQLTQDEWIDI PQGWSQGRTIYGGLVAGLLMHKALSVMNDESKNLLSTSITFVGPVNEGRVRLT
VEILRQGKSVTTI EARLWQDQAVQTILIASFGQPRSSEIFVLNLPEAPDYLSPEQFSRMPFVKMMPECYQQFDLR
WAEGHYPMTQQAPDFGGWCYDIQKHSRALNVADLLILMDIWPPGVLPFQTIAPASSLTWHLTFVVRPVAYELH
DWFKYQVVTQHAAFQYATEYAHLWDAQNRLIAISRQTVTVFA

Figure 31

**Amino acid sequence of hypothetical protein CC2472 of *Caulobacter crescentus* CB15
(protein_id='NP_421275.1)**

MTLYTDLVAAIASTETGFSAHVSDDWKQGRTTYGGLSGALCVEAALRAFPEAPPLRSAQFAFVGPAAGELAISVR
PLRQ GKSTL FVAVDLIGEQGVATHGVLTFGAARTSAISYEEVLCPPVAPAGACELFFPENRQGAPHFSAQFEVRK
AGGTRPLAGGEPEYLLWIRHRDPAATSI SALVALADMPPPPAMALFPQFGPISTMTWSLDIVGLPEADDDGWRL
RTRAETIGDGYSTQEMHLWDAKGRPLVLRQNVAIFV

Figure 32

**Amino acid sequence of hypothetical protein ELI0992 of *Erythrobacter litoralis*
HTCC2594
(protein_id=ZP_00375750.1)**

MSVSDLRAPITSEGGAVTLPADKWLQGRTLFGGASALVAYTAAVRAF PDL PPLRAAQIGFVAPVGKDVET
RAAMVRQGRNVAQVRSELLVEGKVALTAFWLFGTGREANAVHAAAKADPWPGAPEENDSAMTDKGPPFIV
NNFDIRRAQETQGPGEPIVRRWFRLTDRGELDRVSELILVGD TLP PGAMRAMQRQGP I SSINWSFNILDA
ELGTRDGWWLGETASQHAGAGYSSERLRLWNADGVQVMDGLQSVAVFG

Figure 33

**EXTRACELLULAR
POLYHYDROXYALKANOATES PRODUCED
BY GENETICALLY ENGINEERED
MICROORGANISMS**

[0001] The present invention is in the field of biosynthesis of polyhydroxyalkanoates (PHA). The invention relates to a genetically engineered microorganism having at least one gene involved in the metabolism, preferably in the production, of polyhydroxyalkanoates (PHA). This microorganism is useful in commercial production of polyhydroxyalkanoates. The present invention further relates to a method for the production of polyhydroxyalkanoates (PHA).

[0002] Polyhydroxyalkanoates (PHA) are polymers that are biodegradable and biocompatible thermoplastic materials (polyesters of 3-hydroxy fatty acids), produced from renewable resources, with a broad range of industrial and biomedical applications (Williams and Peoples, 1996). Polyhydroxyalkanoates (PHA) are synthesized by a broad range of bacteria and have been extensively studied due to their potential use to substitute conventional petrochemical plastics to protect the environment from harmful effects of plastic wastes.

[0003] PHA can be divided into two groups according to the length of their side chains and their biosynthetic pathways. Those with short side chains, such as PHB, a homopolymer of (R)-3-hydroxybutyric acid units, are crystalline thermoplastics, whereas PHA with long side chains are more elastomeric. The former have been known for about seventy years (Lemoigne and Roukheldman, 1925), whereas the latter materials were discovered relatively recently (deSmet et al., 1983, J. Bacteriol. 154: 870-78). Before this designation, however, PHA of microbial origin containing both (R)-3-hydroxybutyric acid units and longer side chain (R)-3-hydroxyacid units from 5 to 16 carbon atoms had been identified (Wallen, Rohweder, 1974, Environ. Sci. Technol. 8: 576-79). A number of bacteria which produce copolymers of (R)-3-hydroxybutyric acid and one or more long side chain hydroxyacid units containing from 5 to 16 carbon atoms have been identified (Steinbuechel, Wiese, 1992, Appl. Microbiol. Biotechnol. 37: 691-97; Valentin et al., 1992, Appl. Microbiol. Biotechnol. 36: 507-14; Valentin et al., Appl. Microbiol. Biotechnol. 1994, 40: 710-16; Abe et al., 1994, Int. J. Biol. Macromol. 16: 115-19; Lee et al., 1995, Appl. Microbiol. Biotechnol. 42: 901-09; Kato et al., 1996, Appl. Microbiol. Biotechnol. 45: 363-70; Valentin et al., 1996, Appl. Microbiol. Biotechnol. 46: 261-67; U.S. Pat. No. 4,876,331). These copolymers can be referred to as PHB-co-HX (wherein X is a 3-hydroxyalkanoate or alkanate or alkenoate of 6 or more carbons). A useful example of specific two-component copolymers is PHB-co-3-hydroxyhexanoate (PHB-co-3HH) (Brandl et al., 1989, Int. J. Biol. Macromol. 11: 49-55; Amos & McInerney, 1991, Arch. Microbiol. 155: 103-06; U.S. Pat. No. 5,292,860).

[0004] However, although PHAs have been extensively studied because of their potential use as renewable resource for biodegradable thermoplastics and biopolymers (as mentioned above) and have been commercially developed and marketed (Hrabak, O. 1992), their production costs are much higher than those of conventional petrochemical-based plastics, thus presenting a major obstacle to their wider use (Choi and Lee 1997). As described above, many bacteria produce PHA, e.g. *Alcaligenes eutrophus*, *Alcaligenes latus*, *Azotobacter vinlandii*, *Pseudomonas acitophila*, *Pseudomonas oleovorans*, *Escherichia coli*, *Rhodococcus eutropha*, *Chro-*

mobacterium violaceum, *Chromatium vinosum*, *Alcanivorax borkumensis* etc.. All PHA producing bacteria known in the art produce intracellular PHA and accumulate it in PHA granules (Steinbuechel, 1991). The main aspect which renders PHA production expensive and therefore unfavorable as compared to petrochemical-based plastic, is the difficulty to recover produced PHA from within the bacterial cells where it is accumulated. In order to reduce the total production costs of PHA the development of an efficient recovery process was considered to be necessary generally aiming at cell disruption (Lee 1996) by

[0005] i) an appropriate solvent,

[0006] ii) hypochlorite extraction of PHA and/or

[0007] iii) digestion of non-PHA cellular materials.

[0008] However, at an industrial scale, all methods known in the art require large amounts of chemical reagents and/or enzymes which is an obstacle to reducing the recovery cost. Therefore, alternative strategies for PHA recovery are in urgent need.

[0009] The object of the present invention is to provide a system permitting production of commercially significant levels of PHA while reducing the costs for recovering produced PHA.

[0010] This technical problem is solved by the invention, especially in providing a microorganism that complies with the aforementioned requirements and is applicable in said system.

[0011] The invention relates in its first embodiment to a genetically engineered microorganism having at least one modification in at least one gene encoding for a protein involved in the metabolism of polyhydroxyalkanoate(s) (PHA) or, preferably, in at least one gene encoding for a protein, which interferes in the metabolism of the microorganism with the production of PHA, wherein the at least one modification causes extracellular deposition, e.g. excretion, of polyhydroxyalkanoate(s) (PHA), preferably medium or long chain polyhydroxyalkanoate(s) (PHA), produced by the microorganism, preferably in/into the culture medium. Preferably, the microorganism comprises one of the nucleic acid sequences shown in FIGS. 11, 12, 14, 16 und 18 to 25 or a functional fragment or variant thereof. More preferably, the microorganism may comprise nucleic acids No. 180 to No. 680, preferably No. 230 to No. 640, more preferably No. 310 to No. 550, most preferably No. 350 to No. 510, according to one of the nucleic acid sequences shown in FIGS. 11, 12, 14, 16 und 18 to 25, or a functional fragment or variant thereof.

[0012] It has been found by the inventors that a genetically engineered microorganism according to the invention produces extracellular PHA by extracellularly depositing, e.g. excreting PHA (produced intracellularly) in/into the culture medium. The deposition, e.g. excretion of PHA by a microorganism has not been described before in the art. Preferably, the microorganism of the present invention produces large amounts of PHA, preferably overproduces PHA, and deposits a large proportion of its PHA product in the extracellular medium, unlike the microorganisms known in the art. Extracellular deposition, e.g. excretion, and overproduction of large amounts PHA into the culture medium was achieved by modifying at least one gene encoding a polypeptide involved in the metabolism of PHA or, preferably by introducing at least one modification in a gene encoding a polypeptide (enzyme) interfering with the production of PHA. The term "polypeptide" according to the invention also encompasses the terms "peptide", "protein" or "enzyme".

[0013] Various genes encoding polypeptides are involved in the metabolism of PHA. Several of these genes are specified in Table 1 as represented by FIG. 5. Thus, a gene according to the invention encompasses any gene encoding a polypeptide which is involved in PHA metabolism, preferably in PHA production. Preferably, such gene encodes (without being restricted to) PHA synthase, poly(3-hydroxyalkanoate)synthase, enoyl-CoA hydratase, and PHB synthase.

[0014] Other enzymes, which are also involved in the fatty acid metabolism, e.g. fatty acid synthesis or beta-oxidation (poly(3-hydroxybutyrate)depolymerase, acyl-CoA transferase, reductase, or thiolase), may be modified as well. However, these enzymes do not specifically influence PHA synthesis and are, therefore, less preferred for a modification allowing to increase PHA synthesis.

[0015] Preferably, the set of genes required for PHA production is not compromised in a microorganism of the invention. Any modification introduced into any of the PHA production genes (see above) is preferably intended not to reduce, but to enhance their enzymatic activity. Thereby, the microorganism according to the invention is allowed to provide a higher yield of PHA than microorganisms known in the art.

[0016] In contrast, a microorganism according to the invention contains most preferably modifications in any genes encoding for polypeptides which compromise the production of PHA. In particular, a microorganism according to the invention is modified in a gene encoding enzymes which cleave Acyl-CoA molecules by a thioesterase function, e.g. Acyl-CoA thioesterases. Depending on the specific microorganisms one or more Acyl-CoA thioesterases are acting on the fatty acid metabolism. Acyl-CoA thioesterases were especially studied in *E. coli* possessing two of such enzymes called acyl-CoA thioesterase I (encoded by *tesA* gene) and acyl-CoA thioesterase II (encoded by *tesB* gene). Thioesterase I exhibits specificity towards C12 to C18 acyl-CoA esters (Bonner, W M et al., 1972, J. Biol. Chem. 247, 3123-3133), while thioesterase II cleaves C6 to C18 acyl-CoA esters as well as β -hydroxyacyl-CoA esters of chain length C12 to C18 (Barnes et al., 1970, The Journal of Biological Chemistry, vol. 245, No. 12, issue of June 25, 3122-3128). *TesA* is implicated in chain termination of de novo biosynthesis of fatty acids and mediates acyl-ACP intermediates from the fatty acid de novo biosynthesis pathway to fatty acid β -oxidation in *E. coli* (Klinke, S Q et al., 1999, Appl. Environ. Microbiol. 65: 540-548). Up to now, little was known on physiological role of *tesB* in bacterial metabolism.

[0017] A very recent report described that thioesterase II plays an important role in 3-hydroxyalkanoic acid (3-HAA) production (Zweng, Z et al., 2004, Appl. Environ. Microbiol. 70(7): 3807-3813) by cleaving 3-hydroxyacyl-CoA thioester bonds thereby converting them into free 3-HAA. According to the invention, it was found that various microorganisms express thioesterases which act with a high specific activity on the cleavage of 3-hydroxyacyl-CoAs which forms the building block of PHA synthesis. These thioesterases allow to release free 3-HAAs. However, the conversion to 3-HAA is a reaction which competes with the synthesis of PHA by PHA-synthase, which acts on the same cellular intermediate (namely 3-hydroxyacyl-CoAs). According to the invention, it was found that (i) the release of free 3-HAAs and the synthesis of PHA are interfering metabolic pathways and (ii) that functional knock-out of the specific thioesterase, herein

termed *tesB*-like thioesterase, provides for deposition of PHA in the extracellular medium. A functional knock-out mutation in a *tesB*-like gene (as described below in more detail) was identified to increase the intracellular amount of 3-hydroxyacyl-CoA in a number of microorganism, thereby guiding the metabolism of 3-hydroxyacyl-CoA (as PHA precursor) towards PHA synthesis (see FIG. 8).

[0018] As explained above, the present invention is based on the general finding that (knock-out) modifications of thioesterases using (R)-3-OH-acyl-CoA as substrate allow PHA producing microorganisms to deposit PHA in the extracellular medium. While e.g. in *Alkanivorax*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Acinetobacter sp.*, *Caulobacter crescentus* the thioesterase found to act specifically on (R)-3-OH-acyl-CoA, is acyl-CoA thioesterase *tesB*-like, other PHA producing microorganism being based on a slightly different metabolism use other thioesterase, e.g. acyl-CoA thioesterase *tesB* or *tesA*, to produce 3-HAA.

[0019] In any case, a modification of an thioesterase specifically using (R)-3-OH-acyl-CoA to produce 3-HAA is most preferably expected to either reduce the thioesterase activity, e.g. of acyl-CoA thioesterase *tesB*-like, considerably, e.g. by at least 50%, more preferably by at least 60%, even more preferably by at least 80%, more preferably by at least 90%. In a particularly preferred embodiment, the enzymatic activity of this thioesterase, e.g. acyl-CoA thioesterase *tesB*-like, is completely switched off.

[0020] The present invention also encompasses *tesB*-like homologs of proteins as disclosed herein (and as well their encoding nucleic acids), particularly of thioesterases. In the context of the present invention *tesB*-like homologs of proteins as disclosed herein is meant to include any protein or peptide sequence, occurring in a different microorganism than a *tesB*-like homolog of proteins as disclosed herein, which preferably show a (significant) homology to this *tesB*-like protein and display similar or even identical biological function. A (significant) homology to a *tesB*-like protein as shown herein may be easily determined by a skilled person using method well known in the art, e.g. methods for determining sequence identities as also disclosed herein or by activity assays.

[0021] According to the finding of the present invention *Alkanivorax* is characterized by two *tesB* thioesterases, namely acyl-CoA thioesterase *tesB*-like specifically acting on (R)-3-OH-acyl-CoA to produce 3-HAA and acyl-CoA thioesterase *tesB*, which is involved in the production of fatty acids. By performing homology searches (BLAST searches) in several PHA producing bacteria the inventors have screened for other microorganisms showing the same or a similar metabolic structure as in *Alkanivorax* (with a homologue of the highly specific acyl-CoA thioesterase *tesB*-like and another thioesterase (*tesB*)).

[0022] Both types of thioesterases being present in *Alkanivorax borkumensis* SK2 are also expressed in a number of closely related PHA producing gamma-proteobacteria (e.g., *Pseudomonas putida*, *P. aeruginosa*, *P. aeruginosa*, *P. syringae*, *P. fluorescens*, *Idiomarina loihiensis*, *Acinetobacter sp.*, *Caulobacter crescentus*). The homologues of the *Alkanivorax* *tesB*-like protein are named differently in the other microorganisms, e.g. as *tesB*-like thioesterase, putative acyl-CoA thioesterase II or hypothetical protein. However, it is to be understood that the term “*tesB*-like” is intended to encompass all these thioesterases of the aforementioned microorganism

with deviating nomenclature. These homologues of the *Alkanivorax* tesB-like thioesterase are presented in Table 4 (see Table 4 of FIG. 10). The presence of two thioesterases in *Alkanivorax* and the other aforementioned microorganisms, namely the tesB and tesB-like proteins, is explained by the present inventors by different functions of tesB and tesB-like proteins. Most likely, the tesB protein exclusively acts on C6 to C18 acyl-CoA derivatives, whereas the tesB-like protein exclusively cleaves hydroxyacyl-CoAs. This conclusion was supported by an earlier study of tesB protein showing that unlike similar thioesterase II in *E.coli* (Barnes et al., supra), tesB thioesterase II of PHA-producing *Rhodobacter sphaeroides* (Wieczorek, R A et al., 1996, FEMS Microbiology Letters 135: 23-30) is not able to hydrolyze 3-hydroxyacyl-CoA substrates (Seay, T et al., 1982, Biochemistry May 6, 25(9): 2480-2485).

[0023] In a preferred embodiment, the microorganism of the present invention typically contains at least one modified gene as outlined above, wherein the modified gene is integrated into its chromosome.

[0024] The modification of at least one of said genes encoding a protein involved in PHA metabolism and/or, preferably, the degradation of (R)-3-OH-acyl-CoA is achieved by inserting a mutation in the nucleic acid sequence of the gene using genetic engineering techniques. The term “genetically engineered” (or genetically modified) means the artificial manipulation of a microorganism of the invention, its gene(s) and/or gene product(s) (polypeptide). Subsequently, the modification (mutation) was confirmed by sequence analysis (see e.g. nucleic acid and amino acid sequences of FIGS. 11 to 14 and 15 to 33 as well as Examples).

[0025] The term “modification” encompasses any manipulation and mutation of a microorganism of the invention, especially of at least one gene of said microorganism of the invention. Preferably, the modification results in an alteration of the nucleic acid sequence(s) of said at least one gene and is typically expressed on the amino acid sequence level accordingly or may be due to a modification in the regulatory regions, e.g. promotor regions of the gene. Preferably, the modification resulting in an alteration of said nucleic acid sequence(s) is carried out by addition, substitution, deletion or insertion of one or more nucleotide(s). Furthermore, the modification may encompass one or more additional copies of a gene in a microorganism and/or (complete) deletion of a gene. Deletion may also be due to a disruption of the gene by recombination or insertion of e.g. a transposon. In a preferred embodiment, a modification within a microorganism according to the present invention causes a complete or partial inactivation of at least one gene encoding a protein which is involved in the metabolism interfering with the production of PHA (e.g. by biochemically converting intermediates of the PHA synthesis pathway), more preferably a thioesterase, even more preferably a thioesterase, which degrades intermediates of the PHA synthesis pathway, and most preferably a thioesterase, which converts (specifically) (R)-3-OH-acyl-CoA to 3-HAA. In the most preferred embodiment of the present invention the microorganism is defective with regard to the tesB-like thioesterase (*Alkanivorax*) or a homologue thereof (in other microorganisms). The defective character may be due to various modification on the genetic level or may be due to posttranscriptional modifications reducing or abolishing the enzymatic activity of the relevant thioesterase.

[0026] Additionally, modifications in one or more of the genes involved in the PHA synthesis may occur in the micro-

organism according to the invention. These modifications may be directed to PHA synthase, poly(3-hydroxyalkanoate) synthase, enoyl-CoA hydratase, and/or PHB synthase and/or other enzymes involved in the fatty acid metabolism. Genes encoding for enoyl-CoA hydratase in *Alkanivorax* are e.g. ABO_2240; ABO_0526; ABO_1238; ABO_0987; ABO_0148; or ABO_1645. Enoyl-CoA hydratase links beta-oxidation with PHA biosynthesis catalyzing production of 3-hydroxy-acyl-CoA from enoyl-CoA, an intermediate of beta-oxidation. 3-hydroxy-acyl-CoA is a precursor of PHA and is incorporated by phaC synthase to PHA. There are two genes encoding PHA synthases in *Alkanivorax*: ABO_2214 and ABO_1418. PHA synthases catalyze the crucial last step in biosynthesis of PHA. Generally, the enzymatic activity of these enzymes is enhanced due to the modification(s) introduced.

[0027] According to the invention the genetically engineered microorganism has at least one modification in at least one gene encoding a protein which is involved in the metabolism interfering with the production of PHA and, optionally, at least one modification of a gene involved in PHA metabolism. Thus, it is possible that the microorganism has only one modification in one gene encoding a protein which is involved in the metabolism interfering with the production of PHA. However, it is also possible that the microorganism has more than one (two or more) modification(s) in the same gene or in two (or more) different genes involved in the relevant metabolism of the microorganism according to the invention. In this case, it is possible that the more than one modification causes different results or phenotypes. For example, one of these modifications results in PHA excretion whereas another modification results in PHA overproduction (as mentioned below).

[0028] Moreover, it is also possible that the microorganism according to the present invention has more than one modification in different genes with differing functions, namely (at least) one modification occurs in (at least) one gene encoding a protein which is involved in the metabolism interfering with the production of PHA, whereas (at least) one other modification occurs in (at least) one gene which is involved in PHA metabolism. In addition, further genes may be modified, e.g. genes encoding for a protein involved in secretion mechanisms. In such a case, it is also possible that (at least) one of these modifications results in (increased) PHA excretion whereas (at least) one other modification results in PHA overproduction (as mentioned below).

[0029] Several suitable genetic engineering technique known in the art can be used to generate a microorganism of the invention. In general, genes from any source can be broken into pieces and modified in various ways, using microorganisms and their enzymes or transposable elements as molecular tools. According to the present invention, it is even possible to construct at least one completely artificial gene which either involved in the metabolism of PHA by a microorganism of the invention (e.g. increasing the amount of PHA produced) and/or involved e.g. in the inhibition of 3-HAA production using genetic engineering techniques. Once the desired gene has been selected or created, it can be inserted into a microorganism of the invention where it can be expressed to produce the desired gene products. For example, a wide range of genetic engineering methods are based on molecular cloning. In molecular cloning, a DNA fragment from essentially any type of genetic element composed of double-stranded DNA is recombined with a vector and intro-

duced into a suitable host. Commonly employed cloning vectors include, e.g., plasmids and bacteriophages (e.g., plasmid pBR322, bacteriophage lambda, also see below). Molecular cloning can be divided in single steps:

[0030] 1. isolation and fragmentation of the source DNA (e.g. genomic DNA, cDNA, synthetic DNA etc.)

[0031] 2. joining DNA fragments to a cloning vector with DNA ligase and

[0032] 3. introduction and maintenance in a host organism (microorganism), e.g., by transformation.

[0033] Thereby, the microorganism of the invention may e.g. contain an artificial or native gene (operably coupled to a strong promoter), which expresses a protein of the PHA synthesis pathway in excess, thereby increasing the amount of PHA pathway intermediates. As a result, PHA is produced in larger amounts than naturally. The additional gene is inserted into the cell and may be located on a separate DNA molecule, e.g. a vector (e.g. a plasmid) or may be incorporated into the chromosome of the cell.

[0034] Another preferred technique to alter a nucleic acid sequence relates to oligonucleotide site-directed mutagenesis whereby a specific base pair in a gene can be changed to another base pair (see, e.g., Comack B, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, 8.01-8.5.9, Ausubel F, et al., eds. 1991). In this technique an oligonucleotide, whose sequence contains a mutation of interest, is synthesized as described supra. This oligonucleotide is then hybridized to a template containing the wild-type nucleic acid sequence. In a preferred embodiment of this technique, the template is a single-stranded template. Particularly preferred are plasmids which contain regions such as the fl intergenic region. This region allows the generation of single-stranded templates when a helper phage is added to the culture harboring the phagemid. After annealing of the oligonucleotide to the template, a DNA-dependent DNA polymerase is used to synthesize the second strand from the oligonucleotide, complementary to the template DNA. The resulting product is a heteroduplex molecule containing a mismatch due to the mutation in the oligonucleotide. After DNA replication by the host cell a mixture of two types of plasmid are present, the wild-type and the newly constructed mutant. This technique permits the introduction of convenient restriction sites such that the coding nucleic acid sequence may be placed immediately adjacent to whichever transcriptional or translational regulatory elements are employed by the practitioner. The construction protocols utilized for *E. coli* can be followed to construct analogous vectors for other organisms, merely by substituting, if necessary, the appropriate regulatory elements using techniques well known to skilled artisans.

[0035] An especially preferred technique according to the invention relates to transposon mutagenesis, a type of random recombination. This procedure usually involves breakage reactions at the ends of the mobile DNA segments embedded in chromosomes and the attachment of those ends at one of many different non-homologous target DNA sites. It does not involve the formation of heteroduplex DNA. Transposons can be used as mutagenic agents without the use of chemical or physical agents. Transposons (also called transposable elements) can be integrated in the chromosome (e.g. of a bacterium) at various locations and causes mutations (mutations are defined as inherited or artificial changes in the base sequence of nucleic acids and/or in the amino acid sequence of peptides or polypeptides), wherein an insertion within a gene generally results in the loss of gene function. Thus, they

provide facile means of creating mutants throughout the chromosome. The most convenient element for transposon mutagenesis is one containing an antibiotic resistance gene. Clones containing the transposon can then be selected by isolation of antibiotic-resistance colonies. Two transposons widely used for mutagenesis are Tn10, which contains a marker for tetracycline resistance, and Tn5, which confers neomycin and kanamycin resistance. Accordingly, a preferred embodiment relates to a microorganism of the invention, wherein the at least one modification is performed by transposon mutagenesis, preferably based on miniTn5 kanamycin element (miniTn5 Km element) (sequences see FIGS. 14 and 15), more preferably miniTn5 streptomycin element (miniTn Str/Sp element) (see Example 1).

[0036] In a preferred embodiment of the present invention, the microorganism contains a modification, which causes polar effects subsequent to transposon insertion, preferably Tn-5 insertion, on the gene downstream to the modified gene as described below.

[0037] Another useful technique relates to conservative site-specific recombination involving the production of very short heteroduplex joint and therefore requiring a short DNA sequence that is the same on both donor and recipient DNA molecules. In this pathway, breakage and joining occur at two special sites, one on each participating DNA molecule. The double-stranded molecule obtained is inserted into a cloning host by transformation and mutants are selected. Depending on the orientation of the two recombination sites, DNA integration, DNA excision or DNA inversion can occur. Conservative site-specific recombination is especially usable to turn genes on or off.

[0038] As mentioned above, the at least one modification of at least one gene encoding a protein which is involved in the metabolism interfering with the production of PHA and/or a gene encoding a protein involved in PHA metabolism leads to extracellular deposition, e.g. excretion, of polyhydroxyalkanoate(s) (PHA), preferably medium or long chain polyhydroxyalkanoate(s) (PHA), produced by the microorganism, preferably into the surrounding medium. Thus, microorganism of the invention typically deposits, e.g. excretes, polyhydroxyalkanoates (PHA), preferably medium or long chain polyhydroxyalkanoates (PHA), preferably into the surrounding natural or culture medium. According to the invention the term “deposition” or “deposited” means that the microorganism releases the (intracellular) produced PHA, preferably into the surrounding medium which is a culture medium containing all necessary components and suitable conditions (nutrients, buffer, pH, temperature) for existence and growth of the microorganism. The deposition may be due to an active process of living cells (“excretion”) and/or due to the release of PHA from microorganisms dying subsequent to PHA (over)production. A typically polyester of hydroxyacid units (PHA) contains side chain hydroxyacid units [(R)-3-hydroxyacid units] from 5 to 16 carbon atoms. The term “long chain PHA” is intended to encompass PHA containing at least 12, preferably at least 14 carbon atoms per monomer (molecule), whereas 5 to 12 carbon atoms are intended to be meant by “medium chain PHA”.

[0039] According to the invention, overproduction of PHA by the microorganism of the invention has been shown (see FIGS. 1, 4, 6 and 7). Thus, microorganisms having at least one modification of at least one gene encoding a protein which is involved in the metabolism interfering with the production of PHA, in particular a modification leading to a (partially)

defective thioesterase, more particularly to a (partially) thioesterase using (R)-3-OH-acyl-CoA as a substrate with high specificity show generally (in addition to deposition of PHA in the culture medium) an overproduction of polyhydroxyalkanoate(s) PHA, preferably medium and/or long chain polyhydroxyalkanoates (PHA). The term “overproduction” means a PHA production by a microorganism of the invention which is at least 5 times, preferably at least 10 times, more preferably at least 15 times, more preferably at least 25 times, even more preferably at least 40 times, even more preferably at least 50 times, most preferably at least 60 times, most preferably at least 80 times, even most preferably at least 100 times higher than the PHA production of the corresponding wild type microorganism. Wild type microorganism means a PHA producing microorganism which is not genetically engineered and which genes are not artificially modified (mutated). Wild type microorganisms produce normal levels of PHA, but do not show deposition properties. In addition, the microorganism according to invention may contain at least one modification in a gene encoding a protein which is involved in PHA synthesis. This modification may be due to e.g. overexpression of the protein by a modified promoter or another modified regulatory element of the PHA synthesis gene or by additional copies of the PHA synthesis gene (achieved by e.g. transformation of the microorganism) or by a mutation within the coding region of the gene, which increases the activity and/or specificity of the PHA synthesis enzyme.

[0040] In general, the term “microorganism” means a large and diverse group of microscopic organisms that exist as single cells or cell clusters of procaryotic (e.g. bacteria, cyanobacteria) and eucaryotic (e.g. protozoa, fungi) microorganisms. A preferred microorganism according to the present invention is a PHA producing bacterium. The inventive microorganism to be used according to the present invention is preferably selected from the group consisting of PHA producing bacteria *Alcanivorax borkumensis*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Idiomarina loihiensis*, *Acinetobacter sp.*, *Caulobacter crescentus*. Nevertheless, any other PHA producing microorganism, e.g., *Alcaligenes eutrophus*, *Alcaligenes latus*, *Azotobacter vinlandii*, *Pseudomonas acitophila*, *Pseudomonas oleovarans*, *Rhodococcus eutropha*, *Chromobacterium violaceum*, *Chromatium vinosum* may be used as well. Moreover, any microorganism, which does not naturally produce PHA, may be used according to the invention, if such microorganism comprises an expression vector which comprises a gene cluster or a corresponding expression cassette, which allows to express the enzymes needed for PHA production, in particular PHA synthase, poly(3-hydroxyalkanoate)synthase, enoyl-CoA hydratase, and/or PHB synthase, and contains at least one modification as disclosed above. Such an expression vector can be introduced into a microorganism, in particular into a cell of said microorganism, e.g., *Escherichia coli*, by any suitable method, e.g. by transfection, transduction, transformation etc. (see below).

[0041] An especially preferred microorganism according to the present invention is an oligotrophic bacterium, more preferably halophilic oligotroph bacterium, even more preferably marine oil-degrading bacterium, especially of the strain *Alcanivorax*, preferably *Alcanivorax borkumensis*, more preferably *Alcanivorax borkumensis* SK2. *A. borkumensis* is a marine oil-degrading bacterium widely distributed in the aquatic environments. It is a moderately halophilic

oligotroph bacterium which is able to use essentially only petroleum hydrocarbons as carbon and energy sources. *A. borkumensis* is of particular interest because it is mostly found as predominant species in oil polluted sea water (Harayama et al., 1999; Kasai et al., 2001; 2002; Syutsubo et al., 2001) and, accordingly, has key applications for bioremediation purposes. For oligotrophic marine bacteria including *Alcanivorax* oil pollution constitutes temporary conditions of nutrient abundance, characterized by high C/N ratio. Under such conditions the microorganism will store excess of carbon for the use as energy source when lack of nitrogen will not more be a limiting factor. These conditions of high C/N ratio indeed favor microbial intracellular storage inclusions. Correspondingly, carbon excess allows PHA producing bacterial species to be accumulated in form of polyhydroxyalkanoate (PHA) granules (Steinbüchel 1991). Previously, it was described that *A. borkumensis* was not able to produce PHA (Yakimov et al. 1998). However, culturing conditions were not favorable for PHA accumulation due to relatively high nitrogen concentration in the culture medium (5 g/l). According to the invention, the complete genome of *A. borkumensis* was sequenced. Functional analysis of *A. borkumensis* genome revealed the presence of genes encoding proteins for polyhydroxyalkanoate (PHA) production (as shown by homology studies with other organisms). Therefore, according to the invention, it was shown that *A. borkumensis* is a PHA producing bacterium. Further amino acid sequence alignments of the proteins revealed low sequence homology with the proteins involved in PHA synthesis metabolism in other bacterial species (see FIG. 5, Table 1) suggesting a characteristic metabolic root of PHA production in *A. borkumensis*.

[0042] As mentioned above, a microorganism of the present invention may typically contain at least one modified gene encoding a protein interfering with PHA production, preferably a thioesterase, more preferably a thioesterase synthesizing 3-HAA encoding a protein involved in the metabolism of PHA synthesis. However, the invention provides also a nucleic acid sequence which constitutes a gene encoding a protein involved in the metabolism of polyhydroxyalkanoate (s) (PHA), wherein the gene has at least one modification causing excretion of polyhydroxyalkanoate(s) PHA, preferably long chain polyhydroxyalkanoates (PHA), produced by the microorganism of the present invention. Preferably, the modified nucleic acid sequence of the invention is based on a wild type gene selected from the group consisting of PHA synthase, PHB synthase, acyl-CoA transferase, enoyl-CoA hydratase, reductase, thiolase and acyl-CoA thioesterase. On the other hand, the present invention provides a gene modified by at least one modification, preferably rendering the enzymatic activity of the encoded enzyme defective, whereby the gene is acyl-CoA thioesterase tesB-like, preferably acyl-CoA thioesterase tesB-like of *Alcanivorax borkumensis*, more preferably acyl-CoA thioesterase tesB-like of *Alcanivorax borkumensis* SK2 or homologues of this enzyme in other microorganisms, especially as shown by table 4 (FIG. 10).

[0043] An especially preferred microorganism of the present invention has been deposited according to the Budapest Treaty on the International Recognition of the Deposit of

[0044] Microorganisms for the Purpose of Patent Procedure at Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (DSMZ), Mascheroder Weg 1b, 38124 Braunschweig, Germany with the identification reference SK2 C9 mutant and the Accession Number DSM17483.

[0045] A particularly preferred embodiment relates to a nucleic acid sequence comprising or consisting of one of the nucleic acid sequences shown in FIGS. 11, 12, 16 and 18 to 25 or a functional fragment or variant thereof.

[0046] The nucleic acid sequence of the invention can be DNA comprising the coding sequence and eventually further sequences. The nucleic acid molecule can be double stranded or single stranded; single stranded RNA or DNA can be either the coding (sense) strand or the non-coding (antisense) strand. If desired, the nucleic acid sequence can include additional non-coding sequences such as non-coding 3'- and 5'-sequences (including regulatory sequences, for example). All nucleic acid sequences, unless designated otherwise, are written in the direction from the 5' end to the 3' end. The term "nucleic acid sequence" also includes a fragment or variant thereof as described below. Furthermore, the nucleic acid sequence of the invention can be fused to a nucleic acid sequence comprising or consisting of, for example, a marker sequence, a leader sequence or a nucleic acid sequence which encodes a polypeptide to assist, e.g., in isolation or purification of the polypeptide. Representative sequences include, but are not limited to those which encode a glutathione-S-transferase (GST) fusion protein, a poly-histidine (e.g., His6), hemagglutinin, HSV-Tag, for example.

[0047] The term "functional" fragment or variant of a nucleic acid sequence relates to a nucleic acid sequence of the invention which is able to constitute a typically modified gene encoding for a protein involved in the metabolism of PHA, or a gene, encoding for a protein, which interferes in the metabolism of the microorganism with the production of PHA, and/or encodes a biologically active (e.g., a protein involved in the metabolism of PHA, or a protein, which interferes in the metabolism of the microorganism with the production of PHA) polypeptide of the invention as described below.

[0048] Additionally, in the context of the present invention, nucleic acid sequences may be used herein, which encode a protein, which provides for deposition of PHA in the extracellular medium, wherein the protein competes with the synthesis of PHA by PHA-synthase in a microorganism. These nucleic acid sequences may thus be termed herein "dysfunctional" nucleic acid sequences. "Dysfunctional" nucleic acid sequences according to the invention may comprise native nucleic acid sequences according to FIGS. 16 and 18 to 25 or encode native polypeptides sequences according to FIGS. 17 and 26 to 33, wherein these sequences have been interrupted by transposon inserts (e.g. as exemplified in FIGS. 14 and 15) as disclosed above for nucleic acid sequences and the corresponding genes. Furthermore, "dysfunctional" nucleic acid sequences according to the invention may comprises nucleic acid sequences according to FIGS. 11 and 12, which have been interrupted by transposon inserts.

[0049] The term "fragment" of a nucleic acid sequence is intended to encompass a portion of a nucleic acid sequence described herein which is from at least about 25 contiguous nucleotides to at least about 50 contiguous nucleotides, preferably at least about 60 contiguous nucleotides, more preferably at least about 120 contiguous nucleotides, more preferably at least about 180 contiguous nucleotides, more preferably at least about 250 contiguous nucleotides, more preferably at least about 410 contiguous nucleotides or longer in length. In this context, shorter fragments according to the invention are useful as probes and also as primer. Particularly preferred primers and probes selectively hybridize to the nucleic acid sequence encoding the polypeptides described

herein. A primer is a fragment of a nucleic acid sequence which functions as an initiating substrate for enzymatic or synthetic elongation. A probe is a nucleic acid sequence which hybridizes with a nucleic acid sequence of the invention, a fragment or a complementary nucleic acid sequence thereof. Fragments which encode polypeptides according to the invention that retain function as described above are particularly useful.

[0050] Hybridization can be used herein to analyze whether a given fragment or gene corresponds to the microorganism of the invention and thus falls within the scope of the present invention. Hybridization describes a process in which a strand of a nucleic acid sequence joins with a complementary strand through base pairing. The conditions employed in the hybridization of two non-identical, but very similar, complementary nucleic acids varies with the degree of complementarity of the two strands and the length of the strands. Such conditions and hybridization techniques are well known by a person skilled in the art and can be carried out following standard hybridization assays (see e.g., Sambrook J, Maniatis T (2001) supra). Consequently, all nucleic acid sequences which hybridize to the nucleic acid sequence or the functional fragments or functional variants thereof according to the invention are encompassed by the invention.

[0051] A variant of a nucleic acid sequence means a nucleic acid sequence which is derived from a nucleic acid sequence of the invention by addition, substitution, deletion or insertion of one or more nucleic acid(s) retaining the characteristic function of said nucleic acid sequence as described above. Such nucleic acid sequence can exhibit altered properties in some specific aspect (e.g. increased or decreased expression rate). Beside that, skilled artisans will recognize that the amino acids of polypeptides of the invention, as described below, can be encoded by a multitude of different nucleic acid triplets because most of the amino acids are encoded by more than one nucleic acid triplet due to the degeneracy of the genetic code. Because these alternative nucleic acid sequences would encode the same amino acid sequence, the present invention also comprises those alternate nucleic acid sequences.

[0052] A variant of a nucleic acid sequence according to the invention has substantial identity with the nucleic acid sequences described herein. Particularly preferred are nucleic acid sequences which have at least about 30%, preferably at least about 40%, more preferably at least about 50%, even more preferably at least about 60%, yet more preferably at least about 80%, still more preferably at least about 90%, and even more preferably at least about 95% identity with nucleic acid sequences described herein.

[0053] To determine the percent identity of two nucleic acid sequences in the above mentioned context, the sequences can be aligned for optimal comparison purposes (e. g., gaps can be introduced in the sequence of a first nucleic acid sequence). The nucleotides at corresponding nucleotide positions can then be compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between two sequences is a function of the number of identical positions shared by the sequences. Therefore, the determination of percent identity of two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin et al.

(1993), PNAS USA, 90:5873-5877. Such algorithm is incorporated into the NBLAST program which can be used to identify sequences having the desired identity to nucleic acid sequences of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (1997), Nucleic Acids Res, 25:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e. g., NBLAST) can be used. The described method of determination of the percent identity of two can be also applied to amino acid sequences.

[0054] The production of fragments or variants of a nucleic acid sequence of the invention can be carried out following standard methods which are well known by a person skilled in the art (see e.g., Sambrook J, Maniatis T (2001) supra). In general, the preparation of such functional fragments or variants of a nucleic acid sequence can be achieved by modifying (altering) a DNA sequence which encodes a polypeptide of the invention and amplifying the DNA sequence with suitable means, e.g., by PCR technique. Those modifications (mutations) of the nucleic acid sequences may be generated by genetic engineering techniques as described above. The isolation of a functional fragment or functional variant of a nucleic acid sequence can be carried out using standard methods as screening methods (e.g., screening of a genomic DNA library) followed by sequencing or hybridization (with a suitable probe, e.g., derived by generating an oligonucleotide of desired sequence of the target nucleic acid) and purification procedures, if appropriate.

[0055] The present invention also encompasses gene products of the nucleic acid sequence of the invention. Gene products according to the invention not only relate to the transcripts, accordingly RNA, preferably mRNA, but also to alleles, polypeptides or proteins or enzymes, particularly, in purified form. Preferably the gene product is a polypeptide encoded by a nucleic acid sequence of the invention. Preferably, the inventive polypeptide comprises one of the amino acid sequences shown in FIGS. 13, 17 and 26 to 33.

[0056] A “functional” polypeptide according to the invention means that the polypeptide can be used to produce deposited, e.g. excreted, PHA, preferably in combination with other polypeptides involved in the metabolism of PHA. Preferably an overproduction of PHA is also achieved by functional polypeptides of the PHA synthesis pathway. Methods for measuring and analyzing production, excretion and/or overproduction of substances, like PHA, are well known in the art (see e.g., Sambrook J, Maniatis T (2001) Molecular Cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) and are also described by this invention, e.g. Examples 3 to 8, FIGS. 1, 3, 4 to 6). Without being limited thereto, “functional” polypeptides according to the invention may comprises native polypeptides sequences according to FIGS. 17 and 26 to 33.

[0057] Additionally, in the context of the present invention, polypeptides may be used herein, which provide for deposition of PHA in the extracellular medium, particularly by competing with the synthesis of PHA by PHA-synthase in a microorganism, and thus may be termed herein “dysfunctional” polypeptides, e.g. tesB-like proteins as defined herein. Such competition may occur, as explained above, due to thioesterases which act with a high specific activity on the cleavage of 3-hydroxyacyl-CoAs which forms the building block of PHA synthesis. These thioesterases allow to release free 3-HAAs. However, the conversion to 3-HAA is a reac-

tion which competes with the synthesis of PHA by PHA-synthase, which acts on the same cellular intermediate (namely 3-hydroxyacyl-CoAs). As explained above, it was found according to the invention, that (i) the release of free 3-HAAs and the synthesis of PHA are interfering metabolic pathways and (ii) that functional knock-out of the specific thioesterase, herein termed tesB-like thioesterase, provides for deposition of PHA in the extracellular medium. “Dysfunctional” polypeptides according to the invention may comprises native polypeptides sequences according to FIGS. 17 and 26 to 33 or as encoded by nucleic acid sequences according to FIGS. 16 and 18 to 25, wherein these sequences have been interrupted by transposon inserts (e.g. as exemplified in FIGS. 14 and 15) as disclosed above for nucleic acid sequences and the corresponding genes. Furthermore, polypeptides according to the invention may comprises polypeptides sequences as encoded by the nucleic acid sequences according to FIGS. 11 and 12, which have been interrupted by transposon inserts.

[0058] The production of polypeptides of the invention is well known and can be carried out following a number of different standard methods which are well known by a person skilled in the art (see e.g., Sambrook J, Maniatis T (2001) supra), e.g., by solid phase peptide synthesis or recombinant methods. Both methods are described in U.S. Pat. No. 4,617, 149, the entirety of which is herein incorporated by reference. The principles of solid phase chemical synthesis of polypeptides are well known in the art and are described by, e.g., Dugas H. and Penney C. (1981), Bioorganic Chemistry, pages 54-92. For examples, peptides may be synthesized by solid-phase methodology utilizing an Applied Biosystems 430A peptide synthesizer (commercially available from Applied Biosystems, Foster City, Calif.) and synthesis cycles supplied by Applied Biosystems. Essential protected amino acids, such as t-butoxycarbonyl-protected amino acids, and other reagents are commercially available from many chemical supply houses. The isolation of polypeptides of the invention can be carried out using standard methods like separation from the culture medium by centrifugation, filtration or chromatography and precipitation procedures (see, e.g., Sambrook J, Maniatis T (2001) supra).

[0059] The polypeptide(s) of the invention can also be fused to at least one second moiety. The at least second moiety can be an amino acid, oligopeptide or polypeptide and can be linked to the polypeptide of the invention at a suitable position, for example, the N-terminus, the C-terminus or internally. Linker sequences can be used to fuse the polypeptide of the invention with at least one other moiety/moieties. According to one embodiment of the invention, the linker sequences preferably form a flexible sequence of 5 to 50 residues, more preferably 5 to 15 residues. In a preferred embodiment the linker sequence contains at least 20%, more preferably at least 40% and even more preferably at least 50% Gly residues. Appropriate linker sequences can be easily selected and prepared by a person skilled in the art. Additional moieties may be linked to the inventive sequence, if desired. If the polypeptide is produced as a fusion protein, the fusion partner (e.g. HA, HSV-Tag, His6) can be used to facilitate purification and/or isolation. If desired, the fusion partner can then be removed from polypeptide of the invention (e.g., by proteolytic cleavage or other methods known in the art) at the end of the production process.

[0060] The invention also provides a vector comprising the nucleic acid sequence of the invention. The terms “con-

struct”, “recombinant construct” and “vector” are intended to have the same meaning and define a nucleotide sequence which comprises beside other sequences one or more nucleic acid sequences (or functional fragments or functional variants thereof) of the invention. A vector can be used, upon transformation into an appropriate cell (host cell) to cause expression of the nucleic acid. The vector may be a plasmid, a phage particle or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, under suitable conditions, integrate into the genome itself.

[0061] The aforementioned term “other sequences” of a vector relates to the following: In general, a suitable vector includes an origin of replication, for example, Ori p, colE1 Ori, sequences which allow the inserted nucleic acid sequence to be expressed (transcribed and/or translated) and/or a selectable genetic marker including, e.g., a gene coding for a fluorescence protein, like GFP, or a gene which confer resistance to antibiotics, such as the p-lactamase gene from Tn3, the kanamycin-resistance gene from Tn903 or the chloramphenicol-resistance gene from Tn9.

[0062] The term “plasmid” means an extrachromosomal usually self-replicating genetic element. Plasmids are generally designated by a lower “p” preceded and/or followed by letters and numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis or can be constructed from available plasmids in accordance with the published procedures. In addition, equivalent plasmids to those described are known to a person skilled in the art. The starting plasmid employed to prepare a vector of the present invention may be isolated, for example, from the appropriate *E. coli* containing these plasmids using standard procedures such as cesium chloride DNA isolation.

[0063] A vector according to the invention encompasses a (recombinant) DNA cloning vector as well as a (recombinant) expression vector. Preferred vectors according to the invention are *E. coli* pBR322, XL-Blue MRF' and pBK-CMV, bacteriophage lambda etc.. A DNA cloning vector refers to an autonomously replicating agent, including, but not limited to, plasmids and phages, comprising a DNA molecule to which one or more additional nucleic acid sequences of the invention have been added. An expression vector relates to any DNA cloning vector recombinant construct comprising a nucleic acid sequence of the invention operable linked to a suitable control sequence capable of effecting the expression and to control the transcription of the inserted nucleic acid sequence of the invention in a suitable host. Operable linked means that the nucleic acid sequence is linked to a control sequence in a manner which allows expression (e. g., transcription and/or translation) of the nucleic acid sequence. Transcription means the process whereby information contained in a nucleic acid sequence of DNA is transcribed to complementary RNA sequence.

[0064] Aforementioned control sequences are well known in the art and are selected to express the nucleic acid sequence of the invention and to control the transcription. Such control sequences include, but are not limited to a polyadenylation signal, a promoter (e.g., natural or synthetic promoter) or an enhancer to effect transcription, an optional operator sequence to control transcription, a locus control region or a silencer to allow a tissue-specific transcription, a sequence encoding suitable ribosome-binding sites on the mRNA, a sequence capable to stabilize the mRNA and sequences that control termination of transcription and translation. These

control sequences can be modified, e.g., by deletion, addition, insertion or substitution of one or more nucleic acids, whereas saving their control function. Other suitable control sequences are well known in the art and are described, for example, in Goeddel (1990), Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif.

[0065] Promoters for use in bacterial systems also will contain a Shine-Dalgarno sequence operably linked to the DNA encoding the desired polypeptides.

[0066] Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences such as various known variants of SV40 and known bacterial plasmids, e.g., plasmids from *E. coli* including col E1, pBK, pCR1, pBR322, pMb9, pUC 19 and their variants, wider host range plasmids, e.g., RP4, phage DNAs e.g., the numerous variants of phage lambda, e.g., NM989, and other DNA phages, e.g., M13 and filamentous single stranded DNA phages and vectors derived from combinations of plasmids and phage DNAs, such as plasmids which have been modified to employ phage DNA or other expression control sequences. Expression techniques using the expression vectors of the present invention are known in the art and are described generally, for example, in Sambrook J, Maniatis T (2001) supra.

[0067] Preferably, a vector of the invention, especially an expression vector, comprises a gene cluster comprising a modified gene as defined above, e.g. a gene having at least one modification in at least one gene encoding for a protein involved in the metabolism of polyhydroxyalkanoate(s) (PHA) or, preferably, in at least one gene encoding for a protein, which interferes in the metabolism of the microorganism with the production of PHA, wherein the at least one modification causes extracellular deposition, e.g. excretion, of polyhydroxyalkanoate(s) (PHA), preferably medium or long chain polyhydroxyalkanoate(s) (PHA), produced by the microorganism, preferably in/into the culture medium. Such an expression vector can be introduced in any suitable microorganism, as mentioned above, to generate an inventive microorganism producing and excreting PHA. Such gene cluster typically comprises all genes which are necessary or relevant in the metabolism of PHA. Consequently, also encompassed by the invention is an inventive microorganism comprising aforementioned (expression) vector which comprises said gene cluster.

[0068] The invention also provides a cell (also: host cell) comprising a vector or a nucleic acid (or a functional fragment, or a functional variant thereof) according to the invention. A cell (host cell) means a cell of any microorganism described herein which is useful according to the present invention). Furthermore, a cell or host cell means a cell which has the capacity to act as a host and expression vehicle for a nucleic acid or a vector according to the present invention. Preferably, the cell is a prokaryotic cell. Cells comprising (for example, as a result of transformation, transfection or transduction) a vector or nucleic acid as described herein include, but are not limited to, bacterial cells (e.g., *A. borkumensis*, *E. coli*). The choice of a particular cell depends to some extent on the particular expression vector used to drive expression of the nucleic acids of the present invention.

[0069] A vector can be introduced into a cell (host cell) using any suitable method (e.g., transformation, electroporation, transfection using calcium chloride, rubidium chloride, calcium phosphate, DEAE dextran or other substances,

microprojectile bombardment, lipofection, infection or transduction). Transformation relates to the introduction of DNA (nucleic acid sequence) into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integration. Methods of transforming bacterial hosts are well known in the art. Numerous methods, such as nuclear injection, protoplast fusion or by calcium treatment are summarized in Sambrook J, Maniatis T (2001) supra. Transfection refers to the taking up of a vector by a cell (host cell) whether or not any coding sequences are in fact expressed. Successful transfection is generally recognized when any indication or the operation of this vector occurs within the cell.

[0070] Another aspect of the invention relates to a method for producing polyhydroxyalkanoates (PHA) comprising the following steps:

[0071] cultivating a microorganism or a cell of the invention and

[0072] recovering polyhydroxyalkanoates (PHA) from the culture medium.

[0073] Standard methods for cultivating a microorganism or a cell under suitable conditions are well known in the art. See, e.g. below under Examples, Materials and also Sambrook J, Maniatis T (2001) supra.

[0074] PHA can be isolated from the culture medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating or filtrating the components (PHA) of the supernatant, followed by purification, e.g., by chromatographic procedures, e.g., ion exchange chromatography, affinity chromatography or similar art recognized procedures (see also Example 4).

[0075] Another aspect of the invention relates to the use of a microorganism, a polypeptide, a nucleic acid, a vector and/or a cell of the invention for the production and deposition, e.g. excretion, and/or overproduction of polyhydroxyalkanoates (PHA), especially medium and/or long chain polyhydroxyalkanoates (PHA).

[0076] In summary, the invention relates to a genetically engineered microorganism having at least one modification in at least one gene encoding for a protein involved in the metabolism of polyhydroxyalkanoate(s) (PHA) or, preferably, in at least one gene encoding for a protein, which interferes in the metabolism of the microorganism with the production of PHA, wherein the at least one modification causes extracellular deposition, e.g. excretion, of polyhydroxyalkanoate(s) (PHA), preferably medium and/or long chain polyhydroxyalkanoate(s) (PHA), produced by the microorganism, preferably in/into the culture medium. Transposon mutagenesis, based on miniTn5 Str/Sp element (see Example 1) as well as miniTn5 Km element, was used for introducing the at least one modification into at least one gene encoding for a protein, which interferes in the metabolism of the microorganism with the production of PHA. Subsequently, a screening for miniTn5 mutants deficient in biofilm formation, as measured by a deficiency of attachment of mutant cells to plastic surfaces, was performed (see Example 2). A mutant (hereinafter indicated with "C9" or "C9 mutant") was isolated showing a clear deficiency in biofilm formation due to excessive production of secreted polymeric material, later identified as PHA (see Example 4).

[0077] According to the invention production and deposition, e.g. excretion, of PHA, by applying an inventive genetically engineered microorganism, e.g., as derived from *A. borkumensis*, as well as intracellular PHA accumulation in

form of granula by these microorganisms, especially by *A. borkumensis*, was confirmed by chemical analysis. It has been analyzed that by use of these genetically engineered microorganisms, especially *A. borkumensis*, grown e.g., on octadecane under conditions of a high C to N ratio, a mixture of different PHA (hydroxyhexanoate, hydroxyoctanoate, hydroxydecanoate, hydroxydodecanoate) can be produced in form of intracellular granula as storage material. Beyond that, the isolation and characterization of genetically engineered microorganisms having at least one modification, particularly in at least one gene encoding for a protein, which interferes in the metabolism of the microorganism with the production of PHA, wherein the at least one modification causes extracellular deposition, was achieved. This invention will bypass the problem of costly recovery of intracellular produced PHA and will lead to a higher yield of PHA.

[0078] The following Figures and Examples are thought to illustrate the invention. They should not be constructed to limit the scope of the invention thereon. All references cited by the disclosure of the present application are hereby incorporated in their entirety by reference.

[0079] In another embodiment enzymes are provided, which provide for production of 3-HAA as defined above. Such enzymes include, without being limited thereto, any enzymes derived from *Alkanivorax*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Acinetobacter sp.*, *Caulobacter crescentus* the thioesterase and being capable of producing 3-HAA.

[0080] The present invention furthermore provides the use of these enzymes, which provide for production of 3-HAA as defined above, and/or their encoding nucleic acids, for producing PHA, preferably medium or long chain polyhydroxyalkanoate(s) PHA. Such enzymes may be transfected into microorganisms as outlined above, e.g. as a vector or as a (naked)nucleic acid, or in the form of a protein, e.g. as a fusion with cell penetrating peptides, using methods, known in the art.

FIGURES

[0081] FIG. 1 shows PHA production in wild type *A. borkumensis* SK2 (hereinafter also referred to as "SK2" or "SK2 wild type") and mutant *A. borkumensis* SK2 (hereinafter also referred to as "C9 mutant") on either 2% pyruvate or 1.5% octadecane as carbon source. Cells and corresponding supernatants were checked for PHA content, indicated in g/l. The production of Pha was in the following order: C9 mutant supernatant octadecane>C9 mutant supernatant pyruvate>SK2 wild type cells+supernatant octadecane>SK2 wild type cells+supernatant pyruvate. The amount of Pha isolated from C9 mutant cells grown on either pyruvate or octadecane was too low for quantification (third and fifth probe in the diagram). C9 mutants grown on pyruvate produced almost 10 times more PHA than SK2 wild type grown on pyruvate. Moreover, C9 mutants grown on octadecane produced almost 10 times more PHA than SK2 wild type grown on octadecane. Therefore, according to the invention, a genetically engineered microorganism is provided that deposits, e.g. excretes, and overproduces PHA.

[0082] FIG. 2 shows electron microscopic images of ultrathin sections of *A. borkumensis* SK2 strain. Cells were cultivated in ONR7a containing 1.5% (w/v) octadecane and 0.27 g/l of NH₄Cl (storage conditions) and harvested in sta-

tionary phase of growth. These ultrathin sections prepared from the cells confirmed presence of intracellular granula.

[0083] FIG. 3 shows scanning electron microscopy images (FIG. 3A and 3B) and electron microscopic images (FIG. 3C and D) of SK2 wild type cells and C9 mutant cells, grown on Permanox hydrophobic slides covered with octadecane. FIG. 3A and 3C show results for SK2 wild type cells. FIG. 3B and 3D show results for C9 mutant cells confirming the production of extracellular PHA and the excretion of PHA into the surrounding culture medium, respectively. Although both images contain rod-shaped cells, it is obvious that C9 mutant cells are embedded in extracellular network of some deposited, e.g. excreted material, whereas the SK2 wild type cells are not. These results support the hypothesis that C9 mutant strain is involved in the production of some polymeric extracellular material proved by chemical analysis to be PHA. Further EM images of shadow casting technique of SK2 wild type and C9 mutant cells grown on octadecane in storage conditions provided additional support for the hypothesis that the PHA produced by the mutant strain is deposited, e.g. excreted in the culture medium. The EM pictures for the shadow-casted cells of the C9 mutant strain revealed the perforations on the surface of the C9 mutant cells which are probably involved in excretion of intracellular produced PHA.

[0084] FIG. 4 shows in a diagram the comparison of growth characteristics of C9 mutants and SK2 wild type. As can be seen, C9 mutant did have growth characteristics comparable with SK2 wild type when pyruvate was used as a carbon source. In case of octadecane C9 mutant even showed better growth compared with SK2 wild type. SK2 wild type had some deficiency in growth on octadecane in these conditions compared with C9 mutant. The explanation for this could be that the high intracellular PHA content inhibits cell division of SK2 wild type. Cell counts also show the domination in growth on octadecane for C9 mutant (data not shown) which under the conditions of high C/N ratio releases the synthesized PHA into the medium and is still able for cell division. In conclusion, the microorganism according to the invention is absolutely usable for biotechnological, commercial and industrial purposes.

[0085] FIG. 5 shows Table 1 representing the results of in silico analysis of sequences data of the genes for polyhydroxyalkanoate production and mobilization in *A. borkumensis* SK2.

[0086] FIG. 6 shows Table 2 representing the analysis of PHA and its composition.

[0087] To confirm that the substance deposited, e.g. excreted by wild type *A. borkumensis* SK2 (hereinafter also referred to as "SK2" or "SK2 wild type") and mutant *A. borkumensis* SK2 (hereinafter also referred to as "C9 mutant") is in fact PHA, further chemical analysis were conducted and revealed the presence of PHA. The cells were grown on either pyruvate or octadecane as carbon and energy source in PHA accumulating conditions (PHA storage conditions), i.e., high C/N ratio (C:N ratio 100:1) and were separated from the medium. Cells and corresponding supernatants were checked for PHA content. Pha from C9 mutant and SK2 wild type cells or supernatants were purified by sodium hypochlorite digestion and subsequent solvent extraction with acetone/diethylether (Solaiman et al., 1999). The production of Pha was in the following order:

C9 mutant supernatant octadecane>C9 mutant supernatant pyruvate>SK2 wild type cells+supernatant octadecane>SK2 wild type cells+supernatant pyruvate.

[0088] The amount of Pha isolated from C9 mutant cells grown on either pyruvate or octadecane was too low for quantification (see also FIG. 1). SK2 wild type cells grown on octadecane produced almost 3 times more PHA compared with the cells grown on pyruvate (18 mg/l vs. 6.5 mg/l). The amount of PHA isolated from C9 mutant supernatant grown on octadecane produced 22 times more PHA compared with the cells grown on pyruvate (2,960 mg/l vs. 134 mg/l).

[0089] As shown in Table 2, the amount of PHA produced by SK2 wild type was rather low (6.5 mg/l on pyruvate and 18 mg/l on octadecane) and was dependent on the carbon source with more PHA produced on alkanes. The PHA produced by SK2 wild type was consisting of hydroxyhexanoate (C6), hydroxyoctanoate (C8), hydroxydecanoate (C10) and hydroxydodecanoate (C12) with hydroxydecanoate as the dominant monomer compound. Thus, it was shown despite previous findings (Yakimov et al. 1998) that under conditions of high C/N ratio *Alcanivorax borkumensis* SK2 strain produces a mixture of polyhydroxyalkanoates mostly from alkanes. Moreover, later growth of *A. borkumensis* SK2 under high C/N ratio indeed showed granula visible under light microscope consisting of PHA (see also FIG. 2).

[0090] Table 2 also shows that PHA of SK2 wild type were accumulated inside the cells (no extracellular production), whereas the all PHA produced by C9 mutant was deposited, e.g. excreted to the medium cells (extracellular production). The monomer repeat-unit composition and molecular mass of the polymer (from all the batches) were determined by gas chromatography/mass spectroscopic analysis and gel permeation chromatography, respectively.

[0091] FIG. 7 shows Table 3 representing the molecular weights of PHA from *A. borkumensis*. Results for C9 mutant supernatants growing on pyruvate and octadecane and for SK2 wild type growing on pyruvate and octadecane are shown. The *A. borkumensis* SK2 polymer had repeat unit composition ranging from C6 to C12. The molecular weight of the polymers varied from 180,000 to 540,000 Da (up to 2500 monomers per molecule). This corresponds to a monomer repetition of 1,027 to 2,246 units, depending on the polymer. Similar composition and/or molecular weight was observed for C9 mutant, although the PHA molecular weights of the components were slightly lower in SK2 wild type cells. The molecular weight of the monomers also was not dependent on the carbon source.

[0092] These data strongly suggest that the mutation affected the way of PHA production, but not the composition of the produced polymer.

[0093] FIG. 8 shows a scheme of the hypothetical pathway of PHA biosynthesis in *A. borkumensis* SK2 grown on hydrocarbons/pyruvate (Modified version of Klinke et al.

[0094] 1999 Hypothetical pathway of MCL PHA biosynthesis of PHA polymerase- and thioesterase I-containing *E. coli* JMU 193 grown on gluconate). Hydrocarbons are degraded via terminal oxidation step by consequent action of a monooxygenase, alcohol dehydrogenase and aldehyde dehydrogenase resulting in free fatty acids which are activated by acyl-CoA synthase and enter β -oxidation in form of acyl-CoA. The (S)-3-OH-acyl-CoA produced in β oxidation are isomerised into (R)-3-OH-acyl-CoAs by action of an isomerase. Pyruvate enters fatty acid biosynthesis in form of

acetyl-CoA. Acyl-ACP produced in fatty acid biosynthesis is converted to free fatty acids by action of *tesA* and *tesB*. Free fatty acids produced in fatty acid biosynthesis are activated by acyl-CoA synthase and entered beta-oxidation cycle. (R)-3-OH-acyl-CoAs produced in beta-oxidation are used for biosynthesis for either 3-hydroxyalkanoic acids (3-HAA) via action of *tesB*-like acyl-CoA thioesterase and/or polyhydroxyalkanoate acids (PHA) via action of *phaC* synthase. The mutation in the *tesB*-like acyl-CoA thioesterase abolishes production of 3-HAA and results in uncontrolled production of PHA.

[0095] Thus, in other words, according to the invention, the mutation of *tesB*-like gene abolishing release of free 3-HAA from 3-HAA-CoA, would contribute to increasing the pool of the PHA precursor 3-hydroxyacyl-CoA, leading to uncontrolled PHA formation and its following excretion.

[0096] FIG. 9 shows the operon structure of the two genes *tesB*-like and “putative acetyltransferase” forming a single operon.

[0097] The determination of the site of insertion of the transposon (see Example 1) revealed that mini-Tn5 is inserted in acyl-CoA thioesterase *tesB*-like gene (Abo_1044), which is one of the genes which encodes a protein which interferes with metabolism/production/synthesis of PHA, and likely to disrupt function of the gene, thus inactivating the gene. The *tesB*-like gene is followed by a downstream 1-acyl-sn-glycerol-3-phosphate acyltransferase “putative acetyltransferase” gene (Abo_1045). The ORF of “putative acetyltransferase” gene comprises 645 bp, exhibiting an overlap of 3 bp with the last codon of the preceding ORF of *tesB*-like gene and is predicted to encode a 214-amino-acid protein with a predicted molecular mass of 23.7 kDa. *TesB*-like gene and “putative acetyltransferase” gene are arranged in one operon as shown in FIG. 9.

[0098] To assess that the PHA excretion and overproduction phenotype, as described herein, was the result of the modification (mutation) in a gene encoding a protein which interferes with metabolism/production/synthesis of PHA, especially *tesB*-like acyl-CoA thioesterase gene, and to estimate the possibility of polar effect of the transposon mutation on the downstream gene, a site-directed mutant of the downstream gene was constructed and its phenotype compared with C9 mutant (see Example 3). To construct the knockout mutant the wild type copy of this gene was amplified and cloned in a vector that does not replicate in *A. borkumensis*. A Str resistance cassette was inserted into a unique site inside of this gene and the resulted construct was used to replace the wild type copy of the gene. The results were confirmed by light microscopy and chemical analysis of the culture medium (data not shown). They showed that the knockout mutant was deposited, e.g. excreted PHA in the medium which means that the mini-Tn5 insertion had polar effect on “putative acetyltransferase” gene. Therefore, taking into account the known positive contribution of *tesB*-like into PHA production, the PHA excretion and overproduction phenotype can beside *tesB*-like inactivation also be caused by a polar effect of the Tn-5 insertion on the downstream “putative acetyltransferase” gene.

[0099] FIG. 10 represents Table 4 showing acyl-CoA thioesterase proteins of several closely related gram-negative bacteria encoded by genes homologous of the *tesB*-like gene of *Alcanivorax borkumensis* SK2 (Abo_1044). These data are results of corresponding homology searches (BLAST searches) of putative acyl-CoA thioesterase proteins. As can

be seen from Table 4 the *tesB*-like protein nominated differently, namely as putative acyl-CoA thioesterase II, hypothetical protein or *tesB*-like thioesterase. Bacteria of these searches were the species *Pseudomonas putida* KT2440, *Pseudomonas aeruginosa* PA01, *Pseudomonas syringae* pv B728a, *Pseudomonas fluorescens* PfO-1, *Idiomarina loihiensis* L2TR, *Acinetobacter* sp. ADP 1 and *Caulobacter crescentus* CB 15. However, it is to be understood that the terms “*tesB*-like”, “*tesB*-like gene” and/or “*tesB*-like protein” and description thereof is intended to relate to all these mentioned different nominations. The proteins presented in Table 4 show a high homology to the *tesB*-like gene of *A. borkumensis* SK2 (Abo_1044).

[0100] FIG. 11 shows nucleic acid sequences of the two genes *tesB*-like (Abo_1044) (underlined) and “putative acetyltransferase” (Abo_1045) forming a single operon. The first line is the region upstream from the *tesB*-like gene. The start codon of the *tesB*-like gene is atg and marked in bold letters. The Tn5 insertion took place at position 527 within *tesB*-like gene and marked with //. Downstream from the *tesB*-like gene is the “putative acetyltransferase” gene. There is overlap in 3 bp of the end of *tesB*-like (Abo_1044) and the start site of “putative acetyltransferase” (Abo_1045).

[0101] FIG. 12 shows the nucleic acid sequence of *tesB*-like of *A. borkumensis*. The Tn5 insertion took place at position 557 and is indicated with //. Start and stop codons are marked in bold letters.

[0102] FIG. 13 shows the amino acid sequence of *tesB*-like of *A. borkumensis*.

[0103] FIG. 14 shows the nucleic acid sequence of mini Tn5 Km element.

[0104] FIG. 15 shows the amino acid sequence of neomycin phosphotransferase responsible for neomycin and kanamycin resistance.

[0105] FIG. 16 shows the nucleic acid sequence of “putative acetyltransferase” of *A. borkumensis*. Start and stop codons are marked in bold letters.

[0106] FIG. 17 shows the amino acid sequence of “putative acetyltransferase” of *A. borkumensis*.

[0107] FIG. 18 shows the nucleic acid sequence of putative acyl-CoA thioesterase II of *Pseudomonas putida* KT2440.

[0108] FIG. 19 shows the nucleic acid sequence of hypothetical protein PA2871 of *Pseudomonas aeruginosa* PA01.

[0109] FIG. 20 shows the nucleic acid sequence of acyl-CoA thioesterase II, putative, of *Pseudomonas syringae* pv B728a.

[0110] FIG. 21 shows the nucleic acid sequence of acyl-CoA thioesterase of *Pseudomonas fluorescens* PfO-1.

[0111] FIG. 22 shows the nucleic acid sequence of *tesB*-like acyl-CoA thioesterase of *Idiomarina loihiensis* L2TR.

[0112] FIG. 23 shows the nucleic acid sequence of putative acyl-CoA thioesterase II of *Acinetobacter* sp. ADP1.

[0113] FIG. 24 shows the nucleic acid sequence of hypothetical protein CC2472 of *Caulobacter crescentus* CB15.

[0114] FIG. 25 shows the nucleic acid sequence of hypothetical protein ELI0992 of *Erythrobacter litoralis* HTCC2594.

[0115] FIG. 26 shows the amino acid sequence of putative acyl-CoA thioesterase II of *Pseudomonas putida* KT2440.

[0116] FIG. 27 shows the amino acid sequence of hypothetical protein PA2871 of *Pseudomonas aeruginosa* PA01.

[0117] FIG. 28 shows the amino acid sequence of acyl-CoA thioesterase II, putative, of *Pseudomonas syringae* pv B728a.

[0118] FIG. 29 shows the amino acid sequence of acyl-CoA thioesterase of *Pseudomonas fluorescens* PfO-1.

[0119] FIG. 30 shows the amino acid sequence of tesB-like acyl-CoA thioesterase of *Idiomarina loihiensis* L2TR.

[0120] FIG. 31 shows the amino acid sequence of putative acyl-CoA thioesterase II of *Acinetobacter* sp. ADP 1.

[0121] FIG. 32 shows the amino acid sequence of hypothetical protein CC2472 of *Caulobacter crescentus* CB15.

[0122] FIG. 33 shows the amino acid sequence of hypothetical protein ELI0992 of *Erythrobacter litoralis* HTCC2594.

EXAMPLES

Materials: Bacterial Strains, Media and Growth Conditions

[0123] *A. borkumensis* SK2 was used as the wild type for all experiments. The bacteria were grown at 30° C. in modified ONR7a medium (Yakimov et al 1998) where 0.27 g/l of NH₄Cl was used as the source of nitrogen instead of KNO₃. Pyruvate (2%) or octadecane (1.5%) were used as a carbon and energy source.

[0124] *E. coli* was grown at 37° C. in Luria-Bertani medium supplemented with streptomycin (50 µg/ml), chloramphenicol (12.5 µg/ml), kanamycin (50 µg/ml), nalidixic acid (10 µg/ml) as necessary. Plasmids were introduced into *A. borkumensis* by conjugation with *E. coli* strain S17-1.

Example 1

[0125] Mini-Tn5 Mutagenesis

[0126] Transposon mutagenesis was based on miniTn5 Str/Sp element (and miniTn5 Km element) developed by de Lorenzo et al (1998). *A. borkumensis* SK2 was grown at 30° C. on ONR7a media till stationary phase of growth, the cells were centrifuged. The donor and helper cultures of *E. coli* were grown overnight at 37° C. on LB with either streptomycin or chloramphenicol respectively, washed with fresh LB and centrifuged. The pellets of *A. borkumensis* and *E. coli* donor and helper strains were mixed in proportion 4:1:1 and placed on a membrane filter on a plate with LB agar and salts (Na₂HPO₄·2H₂O, 0.45 g/l; NaNO₃, 2.5 g/l; NaCl, 11.5 g/l; KCl, 0.38 g/l; CaCl₂·2H₂O, 0.7 g/l) and 2% 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 the transconjugants were selected on ONR7a with nalidixic acid and streptomycin.

[0127] mini-Tn5 insertion sites of the strains were determined by inverse PCR as described previously (Ochman et al 1988). Shortly, total DNA of mutants was isolated and digested with ClaI which does not cut mini-Tn5 element. The resulted DNA fragments were circularised and the flanking regions of the inserted mini-Tn5 were amplified with two primers: OTR End (GGC CGC ACT TGT GTA TAA GAG TCA G) and 1TR End (GCG GCC AGA TCT GAT CAA GAG ACA G). The conditions for the PCR were: 94° C. for 1.5 min; 48° C. for 1 min; 70° C. for 4 min, 30 cycles. The PCR products were gel purified and used for automate DNA sequencing with BigDye terminators on an ABI Prism 377 sequencer (AP Biosystems). To determine the precise site of transposon mutation for the tesB-like mutant we designed primers which would read the flanking regions of the dis-

rupted gene: 1086F (TTA CTG GCT TCG CAG GAA TGG) and IntSM160 (CTT GGC ACC CAG CAT GCG CGA GCA GG9).

Example 2

[0128] Biofilm Formation Assay

[0129] In order to screen for mini-Tn5 mutants defective in biofilm formation, an assay described by O'Toole and Kolter (1998) was employed. This assay scores the ability of bacterial cells to adhere to the wells of 96-well microtitre dishes made of polyvinylchloride (PVC) plastic. The ONR7A medium (100 µl/well) was inoculated using replicator device. After inoculation, plates were incubated at 30° C. for 48 h, then 25 µl of a 1% solution of crystal violet (CV) was added to each well (this dye stains the cells but not the PVC), the plates were incubated at room temperature for approximately 15 min, rinsed thoroughly and repeatedly with water and scored for the formation of a biofilm.

[0130] Biofilm formation was quantified by the addition of 2×200 µl of 95% ethanol to each CV-stained microtitre dish well. CV-stained biofilm was solubilized in 200 µl of 95% ethanol, of which 125 ml were transferred to a new polystyrene microtitre dish, and the adsorbance was determined with a plate reader at 600 nm (series 700 microplate reader; Cambridge Technology).

Example 3

[0131] Site-Directed Mutagenesis of "Putative Acetyltransferase" Gene

[0132] To analyze, if the phenotype observed with strain C9 was the result of tesB-like inactivation or of polar effect of the mini-transposon mutation on the downstream gene, a targeted "putative acetyltransferase" mutant was constructed. A 769 bp fragment of the downstream "putative acetyltransferase" gene was amplified from *A. borkumensis* SK2 DNA with primers 1087F: (CAGTGATGGCTATGGTCAAAG) and 1087R: (CTTTGATCAGTCCGGCAAAC) and cloned into pCR 2.1 TOPO Cloning vector (Invitrogen) containing ampicillin resistance gene for counterselection. A Str-resistance cassette was excised from Tn5 Str/Sp plasmid (de Lorenzo et al., 1998) and inserted into the unique site inside of the gene. The non-functional "putative acetyltransferase" gene was then re-introduced into *A. borkumensis* SK2 genome by homologous recombination via conjugation. To confirm loss of vector associated DNA Str^R colonies were then plated in parallel on ONR7A agar containing streptomycin, nalidixic acid and ONT7A agar containing ampicillin to identify isolates that had lost the Amp^R marker associated with the TopoCloning vector. The growth characteristics and PHA accumulation of the knockout mutant were measured and compared with the mini-Tn5 mutant and the wild type.

Example 4

[0133] Chemical Analysis of PHA

[0134] To analyze the PHA released into the medium by the wild type *A. borkumensis* SK2 and by the mutant strain, the bacteria were cultured in ONR7a medium containing either 2% of pyruvate or 1.5% octadecane as carbon sources (storage conditions). The bacteria were incubated in a rotary shaker (100 rpm) at 30° C. until late stationary phase of growth. The bacteria were harvested by centrifugation (60'

12,000) and the supernatant and the pellet were collected separately, lyophilized and used for the subsequent chemical analysis of PHA.

Example 5

[0135] PHA Quantification

[0136] Aliquots of the cells and of the supernatant were washed with ice-cold water and dried overnight at 80° C. under vacuum. Polyhydroxyalkanoates (PHA) were purified from the cells by sodium hypochlorite digestion and subsequent solvent extraction with acetone/diethylether (Solamain et al., 1999).

Example 6

[0137] Gas Chromatography/Mass Spectroscopy

[0138] To determine the PHA composition, approximately 2 mg of PHA were let to react in a small flask with a mixture containing chloroforms:methanol:sulphuric acid (1:0.85:0.15 ml) for 2 h at 100° C. in thermostatically regulated bath. This method degrades PHA by methanolysis to its constituent β -hydroxycarboxylic acid methyl esters (FAME). After the reaction, 0.5 ml of distilled water was added and the tube was shaken for 1 min. After phase separation, the organic phase was removed, transferred into a vial and used for analysis. FAMEs were analyzed with gas chromatograph-mass spectrometer (GC/MS, model Varian 3400CX, Varian Chromatography Systems, Sugar Land, Tex., and VG Autospec spectrometer), equipped with a 30 m \times 0.25 mm HP-5 (5% diphenyl and 95% dimethylpolysiloxane) fused silica capillary column; flow rate 1 ml/min; 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 at 0.5 s/scan. The degree of purity of the PHA samples taken for investigation was up to 99.5%. No trace amounts of proteins, carbohydrates and lipids were registered in them. See data in Table 1.

Example 7

[0139] Gel Permeation Chromatography

[0140] The samples of Example 6 were analyzed in a HPLC system with a Spectra-Physics pump and an Aminex HPX-87H column (Bio-Rad, Hercules, Calif.) under the following conditions: column temperature, 50° C.; gradient, isocratic; mobile phase, 5 mM sulfuric acid; flow rate, 0.5 ml/min; detector, light scattering. See data in Table 2.

Example 8

[0141] Electron Microscopy

[0142] Cells were cultivated in ONR7a containing 1.5% (w/v) octadecane and NH₄Cl (storage conditions) and were harvested in the stationary phase of growth. Cells for scanning electron microscopy were grown in the same conditions with the only difference that octadecane was embedded on Permanox cell culture slides (Nalge Nunc). The cells were shadow-casted according to Golyshina et al. (2000), embedding and ultrathin sections were done as described by Yakimov et al. (1998) and scanning electron microscopy was performed as described by Lünsdorf et al. (2001).

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 SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 31

<210> SEQ ID NO 1

<211> LENGTH: 1546

<212> TYPE: DNA

<213> ORGANISM: Alcanivorax borkumensis

<220> FEATURE:

<223> OTHER INFORMATION: Nucleotide sequences of the genes tesB-like (Abo_1044) and plsC (Abo_1045) consisting of an operon in Alcanivorax borkumensis (fig. 11)

<220> FEATURE:

<221> NAME/KEY: misc_feature

<222> LOCATION: (1)..(60)

<223> OTHER INFORMATION: Region upstream from the tesB-like gene

<220> FEATURE:

<221> NAME/KEY: misc_feature

<222> LOCATION: (61)..(63)

<223> OTHER INFORMATION: Start codon of tesB-like gene

<220> FEATURE:

<221> NAME/KEY: gene

<222> LOCATION: (61)..(847)

<223> OTHER INFORMATION: tesB-like acyl-CoA thioesterase (Abo_1044)

<220> FEATURE:

<221> NAME/KEY: misc_feature

<222> LOCATION: (844)..(847)

<223> OTHER INFORMATION: 4 nucleotides overlap of the two genes

<220> FEATURE:

<221> NAME/KEY: misc_feature

<222> LOCATION: (844)..(846)

<223> OTHER INFORMATION: Start codon of plsC

<220> FEATURE:

<221> NAME/KEY: gene

<222> LOCATION: (844)..(1547)

<223> OTHER INFORMATION: plsC (Abo_1045)

<400> SEQUENCE: 1

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ggcttcgcag gaatggtaaa taaagcaccg caaattcaaa acacgcagaa gtgagagacc      60
atgacattcg atgagattct agccacgatt gatggccagg gtaacgccac gtttccagaa      120
gggtgggggc agggccggac cttatttggg ggctgggtgg gggcggtgct gtttgaacat      180
ttagaaaaaa ccgtggctcg cgggagggtt ctacgtagtt tttctctctc ttttgcgccc      240
cctgcggtgc cgggtccggt ggcactagac gagactgtgt ttcggaagg caaatccggt      300
atgcaggcca tgggtctccg ccgtaagga gggcagggtg tggcggttat gttggccagc      360
tttggggcca gtcgccaatc cagtgtagt gtagaagggc catcgcccc agtgatgaaa      420
tctccagagc agagtatttc ggtaccgttt atcaaaggat tgacgccgga ttttttttcc      480
cattttaata tccattacgc agaaggcatg ccgccgttca gtggcagttc tgagcccgat      540
tacggtggtt acatggggtt tactgtgccc ccggaacca tgagcaccgc agcgctgatt      600

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gcattggtgg atacctgggc gccttcagtt ttacctctgc tcaaagggcc agcaccgcc 660
agttccttga cctggaccat ggagctcttg gatgacccta gtgtccactc gccggagact 720
ttatggcaat atcgggtaaa cacggaccaa tgcagtgatg gctatggtca aagtcaggcg 780
gtggtttggg atgctgcggg taaggctgtg gcgttgagtc gacaaacctt tacggtatth 840
gcatgaaatc ggaactggtg ccaactcacgg cgcggacttt aggtaagcaa gtgcctcgtc 900
gtgggcactg gctggtggcg gcgctagggc ggctgatttt gactgttatg gggtgggcgta 960
ttgttggtga tttgcccgat acgctcggg cagttttggc ggtggcgcca cacacgtcga 1020
acatagacgg ggtcattggt atcagtgcta ttcagtcttt gcgcttgat gtgcgcttca 1080
tgggtaagca cacgttgttt aaaggctcgtc ttgggcggtt catgtactgg ctgggaggca 1140
tcctgtgaa tcgagaaagt gccagggatg tggtggaaca gacgacgtcg gtgatggggg 1200
aaacaccatt ttggcttggg ctaacgccgg aggggacggc taaaggcgcc aagcgttggg 1260
aaaccggggt ttaccgtatt gctgagcaaa tgcaggtgcc gattgtcgtg ttaggtttct 1320
gttaccggcg ccggcaggtc cggattgtag attgctttct gccgacgggc gatattgatg 1380
ctgatatggc gcgaatgacc gcgtcgttgg cggatattgt tccgcgcaa cctgcgcagt 1440
tatccgcccc gctgaaagcg gaaaaagctg ctgcggcat tgattgattc agacgttggc 1500
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<210> SEQ ID NO 2
<211> LENGTH: 786
<212> TYPE: DNA
<213> ORGANISM: Alcanivorax borkumensis
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of tesB-like acyl-CoA
thioesterase from
Alcanivorax borkumensis (Abo_1044) (fig. 12)
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(3)
<223> OTHER INFORMATION: Start codon
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (785)..(787)
<223> OTHER INFORMATION: Stop codon
<400> SEQUENCE: 2

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atgacattcg atgagattct agccacgatt gatggccagg gtaacgccac gtttccagaa 60
gggtgggggc agggccggac cttatthggg ggctgggtgg gggcggtgct gtttgaacat 120
ttagaaaaaa ccgtggctcg cgggaggttt ctacgtagtt tttctctctc ttttgcgcc 180
cctgcggtgc cgggtccggt ggcactagac gagactgtgt ttcgggaagg caaatccggt 240
atgcaggcca tggctctccg ccgtaagga gggcagggtg tggcggttat gttggccagc 300
tttggggcca gtcgccaatc cagtgtagtg gtagaagggc catcggcccc agtgatgaaa 360
tctccagagc agagtatttc ggtaccgttt atcaaaggat tgacgccgga ttttttttcc 420
cattttaata tccattacgc agaaggcatg ccgcccgtca gtggcagttc tgagcccgat 480
tacggtgggt acatgggggt tactgtgccc ccggaaacca tgagcaccgc agcgtgatt 540
gcattggtgg atacctgggc gccttcagtt ttacctctgc tcaaagggcc agcaccgcc 600
agttccttga cctggaccat ggagctcttg gatgacccta gtgtccactc gccggagact 660
ttatggcaat atcgggtaaa cacggaccaa tgcagtgatg gctatggtca aagtcaggcg 720

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gtggtttggg atgctgcggg taaggctgtg gcgttgagtc gacaaacctt tacggatttt 780
gcatga 786
```

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<210> SEQ ID NO 3
<211> LENGTH: 261
<212> TYPE: PRT
<213> ORGANISM: Alcanivorax borkumensis
<220> FEATURE:
<223> OTHER INFORMATION: Amino acid sequence of tesB-like acyl-CoA
thioesterase (figure 13)
```

```
<400> SEQUENCE: 3
```

```
Met Thr Phe Asp Glu Ile Leu Ala Thr Ile Asp Gly Gln Gly Asn Ala
1          5          10          15
Thr Phe Pro Glu Gly Trp Gly Gln Gly Arg Thr Leu Phe Gly Gly Leu
20          25          30
Val Gly Ala Val Leu Phe Glu His Leu Glu Lys Thr Val Ala Arg Gly
35          40          45
Arg Phe Leu Arg Ser Phe Ser Leu Ser Phe Val Ala Pro Ala Val Pro
50          55          60
Gly Pro Val Ala Leu Asp Glu Thr Val Phe Arg Glu Gly Lys Ser Val
65          70          75          80
Met Gln Ala Met Val Ser Ala Arg Gln Gly Gly Gln Val Val Ala Val
85          90          95
Met Leu Ala Ser Phe Gly Ala Ser Arg Gln Ser Ser Val Val Val Glu
100         105         110
Gly Pro Ser Ala Pro Val Met Lys Ser Pro Glu Gln Ser Ile Ser Val
115         120         125
Pro Phe Ile Lys Gly Leu Thr Pro Asp Phe Phe Ser His Phe Asn Ile
130         135         140
His Tyr Ala Glu Gly Met Pro Pro Phe Ser Gly Ser Ser Glu Pro Asp
145         150         155         160
Tyr Gly Gly Tyr Met Gly Phe Thr Val Pro Pro Glu Thr Met Ser Thr
165         170         175
Ala Ala Leu Ile Ala Leu Val Asp Thr Trp Ala Pro Ser Val Leu Pro
180         185         190
Leu Leu Lys Gly Pro Ala Pro Ala Ser Ser Leu Thr Trp Thr Met Glu
195         200         205
Leu Leu Asp Asp Pro Ser Val His Ser Pro Glu Thr Leu Trp Gln Tyr
210         215         220
Arg Val Asn Thr Asp Gln Cys Ser Asp Gly Tyr Gly Gln Ser Gln Ala
225         230         235         240
Val Val Trp Asp Ala Ala Gly Lys Ala Val Ala Leu Ser Arg Gln Thr
245         250         255
Phe Thr Val Phe Ala
260
```

```
<210> SEQ ID NO 4
<211> LENGTH: 2356
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Nucleic acid sequence of miniTn5 Km element,
coding repeats functional in transposition and
neomycin phosphotransferase (figure 14)
<220> FEATURE:
<221> NAME/KEY: misc_feature
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<222> LOCATION: (1)..(19)
<223> OTHER INFORMATION: Tn5 I end, functional in transposition
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2338)..(2356)
<223> OTHER INFORMATION: Tn5 O end, functional in transposition

<400> SEQUENCE: 4

ctgtctcttg atcagatctg gccacctagg ccgaattccc ggggatccgg tgattgattg      60
agcaagcttt atgcttgtaa accgttttgt gaaaaaattt taaaataaa aaaggggacc     120
tctagggtcc ccaattaatt agtaataata tctattaaag gtcattcaaa aggtcatcca     180
ccggatcacc ttaccaagcc ctgctagat tgtaaatgcg gatggtgca ttacttcgcc     240
caactattgc gataacaaga aaagcgcctt tcatgatata tctcccaatt ttgtgtaggg     300
cttattatgc acgcttaaaa ataataaaag cgacttgacc tgatagtttg gctgtgagca     360
attatgtgct tagtgcactc aacgcttgag ttaaccgcgc cgcaagcgg cgtcggcttg     420
aacgaattgt tagacattat ttgccgacta ccttgggtgat tcgccttca cgtagtggac     480
aaaatcaacc aactgatctg cgcgagcttc acgctgccgc aagcatcagg gcgcaagggc     540
tgctaaagga agcggaacac gtagaaagcc agtccgcaga aacggtgcta ccccgatga     600
atgtcagcta ctgggctatc tggacaaggg aaaacgcaag cgcaaagaga aagaggtagc     660
ttgcagtggg cttacatgac gatagctaga ctgggcggtt ttatggacag caagcgaacc     720
ggaattgcca gctggggcgc cctctggtaa ggttgggaag ccctgcaaag taaactggat     780
ggctttcttg ccgccaagga tctgatggcg caggggatca agatctgatc aagagacagg     840
atgaggatcg tttcgcatga ttgaacaaga tggattgcac gcaggttctc cggccgcttg     900
ggtggagagg ctattcggct atgactgggc acaacagaca atcggctgct ctgatgccgc     960
cgtgttcggg ctgtcagcgc aggggcgccc ggttcttttt gtcaagaccg acctgtccgg    1020
tgccctgaat gaactgcagg acgagggcgc gcggctatcg tggctggcca cgacgggctg    1080
tccttgogca gctgtgctcg acgttgtcac tgaagcggga agggactggc tgctattggg    1140
cgaagtgccg gggcaggatc tcctgtcatc tcaccttget cctgccgaga aagtatccat    1200
catggctgat gcaatgcggc ggctgcatac gcttgatccg gctacctgcc cattcgacca    1260
ccaagcgaaa catcgcatcg agcagcacg tactcggatg gaagcggtc ttgtcgatca    1320
ggatgatctg gacgaagagc atcaggggct cgcgccagcc gaactgctcg ccaggctcaa    1380
ggcgcgatg cccgacggcg aggatctcgt cgtgacccat ggcgatgcct gcttgccgaa    1440
tatcatggtg gaaaatggcc gcttttctgg attcatcgac tgtggccggc tgggtgtggc    1500
ggaccgctat caggacatag cgttggctac ccgtgatatt gctgaagagc ttggcggcga    1560
atgggctgac cgcttcctcg tgctttacgg tatcgccgct cccgattcgc agecctcgc    1620
cttctatcgc cttcttgacg agttcttctg agcgggactc tggggttcga aatgaccgac    1680
caagcgacgc ccaacctgcc atcacgagat ttcgattcca ccgccgctt ctatgaaagg    1740
ttgggcttcg gaatcgtttt ccgggacgcc ggctggatga tcctccagcg cggggatctc    1800
atgctggagt tcttcgccc accccggctc gatccctcgc cgagttggtt cagctgctgc    1860
ctgaggctgg acgacctcgc ggagttctac cggcagtgca aatccgctcg catccaggaa    1920
accagcagcg gctatccgcg catccatgcc cccgaactgc aggagtgggg aggcacgatg    1980
gccgctttgg tcgaccgga cgggacggat cagtgagggt ttgcaactgt gggtaagga    2040

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tctggatttc gatcacggca cgatcatcgt cgggagggca agggctcaa ggatcgggcc 2100
ttgatgttac cgagagcttg gtaccagtc tgtgtgagca ggggaattga tccggtggat 2160
gaccttttga atgaccttta atagattata ttactaatta attggggacc ctagagggcc 2220
ccttttttat tttaaaaatt ttttcacaaa acggtttaca agcataaagc ttgctcaatc 2280
aatcacggga tccccgggaa ttcgtcgaca agctgcggcc gcctaggccg tggccgaact 2340
tgtgtataag agtcag 2356

```

```

<210> SEQ ID NO 5
<211> LENGTH: 264
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Amino acid sequence of neomycin
phosphotransferase
responsible for neomycin and kanamycin resistance
(figure 15)

```

```

<400> SEQUENCE: 5

```

```

Met Ile Glu Gln Asp Gly Leu His Ala Gly Ser Pro Ala Ala Trp Val
1           5           10           15
Glu Arg Leu Phe Gly Tyr Asp Trp Ala Gln Gln Thr Ile Gly Cys Ser
20           25           30
Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu Phe
35           40           45
Val Lys Thr Asp Leu Ser Gly Ala Leu Asn Glu Leu Gln Asp Glu Ala
50           55           60
Ala Arg Leu Ser Trp Leu Ala Thr Thr Gly Val Pro Cys Ala Ala Val
65           70           75           80
Leu Asp Val Val Thr Glu Ala Gly Arg Asp Trp Leu Leu Leu Gly Glu
85           90           95
Val Pro Gly Gln Asp Leu Leu Ser Ser His Leu Ala Pro Ala Glu Lys
100          105          110
Val Ser Ile Met Ala Asp Ala Met Arg Arg Leu His Thr Leu Asp Pro
115          120          125
Ala Thr Cys Pro Phe Asp His Gln Ala Lys His Arg Ile Glu Arg Ala
130          135          140
Arg Thr Arg Met Glu Ala Gly Leu Val Asp Gln Asp Asp Leu Asp Glu
145          150          155          160
Glu His Gln Gly Leu Ala Pro Ala Glu Leu Phe Ala Arg Leu Lys Ala
165          170          175
Arg Met Pro Asp Gly Glu Asp Leu Val Val Thr His Gly Asp Ala Cys
180          185          190
Leu Pro Asn Ile Met Val Glu Asn Gly Arg Phe Ser Gly Phe Ile Asp
195          200          205
Cys Gly Arg Leu Gly Val Ala Asp Arg Tyr Gln Asp Ile Ala Leu Ala
210          215          220
Thr Arg Asp Ile Ala Glu Glu Leu Gly Gly Glu Trp Ala Asp Arg Phe
225          230          235          240
Leu Val Leu Tyr Gly Ile Ala Ala Pro Asp Ser Gln Arg Ile Ala Phe
245          250          255
Tyr Arg Leu Leu Asp Glu Phe Phe
260

```

-continued

<210> SEQ ID NO 6
 <211> LENGTH: 645
 <212> TYPE: DNA
 <213> ORGANISM: Alcanivorax borkumensis
 <220> FEATURE:
 <223> OTHER INFORMATION: Nucleotide sequence of putative acyltransferase plsC (Abo_1045) (figure 16)
 <220> FEATURE:
 <221> NAME/KEY: misc_feature
 <222> LOCATION: (1)..(3)
 <223> OTHER INFORMATION: Start codon
 <220> FEATURE:
 <221> NAME/KEY: misc_feature
 <222> LOCATION: (643)..(645)
 <223> OTHER INFORMATION: Stop codon

<400> SEQUENCE: 6

```

atgaaatcgg aactggtgcc actcacggcg cggacttttag gtaagcaagt gcctcgtcgt      60
gggcactggc tgttgccggc gctagggcgg ctgattttga ctggtatggg gtggcgtatt      120
gttggtgatt tgcccatac gcctcgggca gttttggcgg tggcgccaca cacgtcgaac      180
atagacgggg tcattggtat cagtgtctatt cagtctttgc gcttggatgt gcgcttcatg      240
ggtaagcaca cgttgtttaa aggtcgtctt gggcggttca tgtactggct gggcgccatc      300
cctgtgaatc gagaaagtgc cagggatgtg gtggaccaga cgacgtcggg gatgggggaa      360
acaccatttt ggcttgggct aacgccggag gggacgcgta aaggcgccaa gcgcttgaaa      420
accgggtttt accgtattgc tgagcaaatg caggtgccga ttgtcgtgtt aggtttctgt      480
taccggcgcc ggcaggtccg gattgtagat tgctttctgc cgacgggcca tattgatgct      540
gatatggcgc gaatgaccgc gtcggtggcg gatattgttc cgcgcaaacc tgccgagtta      600
tccgccccgc tgaagcgga aaaagctgct cgcggcattg attga                          645

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<210> SEQ ID NO 7
 <211> LENGTH: 214
 <212> TYPE: PRT
 <213> ORGANISM: Alcanivorax borkumensis
 <220> FEATURE:
 <223> OTHER INFORMATION: Amino acid sequence of putative acyltransferase plsC (figure 17)

<400> SEQUENCE: 7

```

Met Lys Ser Glu Leu Val Pro Leu Thr Ala Arg Thr Leu Gly Lys Gln
1           5           10           15
Val Pro Arg Arg Gly His Trp Leu Leu Ala Ala Leu Gly Arg Leu Ile
20           25           30
Leu Thr Val Met Gly Trp Arg Ile Val Gly Asp Leu Pro Asp Thr Pro
35           40           45
Arg Ala Val Leu Ala Val Ala Pro His Thr Ser Asn Ile Asp Gly Val
50           55           60
Ile Gly Ile Ser Ala Ile Gln Ser Leu Arg Leu Asp Val Arg Phe Met
65           70           75           80
Gly Lys His Thr Leu Phe Lys Gly Arg Leu Gly Arg Phe Met Tyr Trp
85           90           95
Leu Gly Gly Ile Pro Val Asn Arg Glu Ser Ala Arg Asp Val Val Asp
100          105          110
Gln Thr Thr Ser Val Met Gly Glu Thr Pro Phe Trp Leu Gly Leu Thr
115          120          125
Pro Glu Gly Thr Arg Lys Gly Ala Lys Arg Trp Lys Thr Gly Phe Tyr
130          135          140

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gccagtttcg gcgtegcccc cgcatcggcg gtggcgggtgg aagcgttgcc ggcggccggg 360
atgaagggcc ccgaagagag ccaggagctg ccctatatcc gtaacgtgac cccggagttc 420
actcgctaca tcgcatgcg ctgggcagtg ggcggcctgc cgttctcttc gaacaagtcg 480
cgccagatgg gcggttgat gcgtttccgc gacgaacccg agggagagcc catggaggtt 540
tcccacctgc tggcgctgct cgactcctgg ccgcccggcg tgttgccgca cctgggcacc 600
ccggcgatgg ccagctcgt gacctggacc gccagttcc tccagccgct gccgcagcaa 660
ggcagcggcg actggtgccg ttacctggcg gagatcgagg aggcgctga cggctacggc 720
cacgtggcgg cgcgatgtg gagcggcgac ggccagttgc tggcgatcag ccggcagatg 780
gtcacggtgt tcggctga 798

```

<210> SEQ ID NO 10

<211> LENGTH: 798

<212> TYPE: DNA

<213> ORGANISM: *Pseudomonas syringae* pv B728a

<220> FEATURE:

```

<223> OTHER INFORMATION: Nucleotide sequence of acyl-CoA thioesterase
II,
putative of p. syringae pv B728a (GeneID:3367571)
(figure 20)

```

<400> SEQUENCE: 10

```

atgacctttt ctgaactgat cgatgcgctg cgtcgcgac cgcgctctgt cacgatccct 60
gccgaatggt ctcaagggcg tgccctgctc ggccggctga tggctgcgct gacctacgaa 120
gcgatgcgct cacaggtgcc ggaagggcgg ccggttcggt cgttggcgat cacctttgtc 180
gggccggccg cgcccgggtg gccgattgct ttcgaggtcg acacctgcg ccatggcaag 240
gcagtcagtc aggtgctggg acgcgccatg cagaacggtc aggtcatgac cctgatacaa 300
ggcagcttcg gtgcccctcg ggaatcgatg atcaccgctg ccgagagggc cgcgccggtc 360
cttaaaccgg ttgatcaatg tccggagctg ccgctcgcca gggcgctgat gcctgattac 420
ctgcgcttca tggacatccg ctgggcgctg ggccgcatgc cattcagtaa taccgatca 480
ccggcgattg gcggtacgt gcgctttcgc gatacgcgc acgccacgcc catgagcgaa 540
gcgcacattc tggcgctggt ggacacctgg ccgctgcgg tactgcccga cctggacaaa 600
ccggcccccg gcagctcact gacctggacc attgagttcg tccagcccca gccgtcgctc 660
gataccctgc agtggcgag ctaccgcgca gtcacgagc atgcccgcga tggctatggc 720
cataccgccc cgccattgtg gagccccgac ggcgagctga tcgcaatcag ccgccagacg 780
gttaccgtat ttggctga 798

```

<210> SEQ ID NO 11

<211> LENGTH: 795

<212> TYPE: DNA

<213> ORGANISM: *Pseudomonas fluorescens* Pf0-1

<220> FEATURE:

```

<223> OTHER INFORMATION: Nucleotide sequence of acyl-CoA thioesterase
(locus_tag=Pflu02003109) (figure 21)

```

<400> SEQUENCE: 11

```

atgcgctttt gcgatctgat cgatgctgct cgtcgtcaac cggaggtcac gattccggcg 60
gagtggggcc agggccggc cagttttggc gggctggtgg ccgcgctgca atttgaagtg 120
atgcgcacca aggttccgac cgatcggccc gtacgttcgc tggcgatcac cttcgtcggc 180
ccggtcgagc ccgaagtgcc ggtgagtttt gaagtcgagg tactacgca aggcaaagcg 240

```


-continued

```

gtcagccagg tgctggggcg tgctgtgcag aacggtcagg tggtgacgat ggtgcaaggc 300
agcttcgggg cttecgggcc atcggaagtg gcggttgaag cctatcccgc gccggaaatg 360
aagcactggg acgattgcca ggaactgccg tacatcaaag gcgtaacccc cgagttcatg 420
cgtcatctgg cgatgcgctg gagcggttggc gggatgcccgt tcaccggcaa tcaatcgcg 480
ctgatgggtg gctgggtgcg cctgcgtggg gatgtgaagg aagagtcggt caacgaagcg 540
cacctgctgg cgctggtcga tgccctggcca ccagcgcgtg tgccgtacct gaagaaaccg 600
gcaccgggca gtacgctgac ctggaccatc gaattcgttc agccgttacg cgatttgagt 660
acgctggatt tttgccaata cctggcggac atcgagtatg ccgccgacgg ttacggccac 720
gtcgcggcca agctgtggag tgcaagggt gaactgattg ccatgagtcg gcagacgggtg 780
acgatcttcg cctga 795

```

```

<210> SEQ ID NO 12
<211> LENGTH: 795
<212> TYPE: DNA
<213> ORGANISM: Idiomarina loihiensis L2TR
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of tesB-like acyl-CoA
thioesterase (locus_tag="IL0656") (figure 22)

```

```

<400> SEQUENCE: 12
atgaacttcc acacagctgt agaacaaatc gttgcggata agaacaatca agtcattgaa 60
ctaccttcg gctgggctca gggacgcgcg tttttcggcg gattcagcgg agcattggct 120
gctcagtttt tgttgaaca atttccgatt gaatatcatc ttcgttccat gagtatctct 180
tttgtcgcgc ctgctgaacc gggtgaggct gagttaaatt accgaatttt gcgcgagggga 240
aaatcggtta ttcaggttgc tgtcgaactg cagcagcaag ggcagattat gttgtcttgt 300
ctggcgagcc tgggcaaagg ccgaagttca acggttacag tggtaagtga aacgccaccc 360
gatcttaaaa ccatcaacga cggaccaggt ttacctgaag cggatattgt cccagagttt 420
gcgaaaaact tegattaccg tattacgtca ggtggtatgc catttagcgg acaaccggga 480
agaacttttg gtggctggat acgttttcgt gaagaacaac agccgctgac aacggcaact 540
atactggctt tagttgacgc ctggccaccg gcagtcttgc ctcatctgga cagccctgcg 600
ccggcctcgt ctttaacctg gactattgag tttcccgata ttctctaca aagtttcagt 660
agccacgact ggtttcagta cgaagctttt attgagcatg ctgaaaatgg ttatgggcac 720
agccgcgagg gtctgtggag tgagaagggc gagttattag ctataagtcg acagactttt 780
acggtatttg cgtaa 795

```

```

<210> SEQ ID NO 13
<211> LENGTH: 804
<212> TYPE: DNA
<213> ORGANISM: Acinetobacter sp. ADP1
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of putative acyl-CoA
thioesterase II (locus_tag="ACIAD0341") (figure 23)

```

```

<400> SEQUENCE: 13
atgagtgatg ctatgccttt agatcagaca ttggaacaac tgacgcaaga tgaatggatt 60
gatattcccc aaggctggtc gcaggaaga acgatttatg gtggattggt tgcaggcttg 120
ctcatgcata aagcattaag tgtgatgaat gatgagtcga aaaatctggt aagtaccagc 180

```

-continued

```

attacttttg tgggaccagt gaatgaaggg cgggtacgac taacagttga aatcttgagg 240
cagggtaaat ctgtcaccac aattgaggca cgtttatggc aggaccaggc tgttcaaacc 300
atcttgatcg caagtttcgg acagccgctg tcttctgaaa tttttgtgct taacttaccc 360
gaggcaccag actatattgtc tcccagagcag ttctcaagaa tgccttttgt aaaaatgatg 420
ccagaatgct atcaacaatt cgatctgcca tgggcagaag gtcattatcc catgactcaa 480
caagctccag attttggtgg ctggtgccgt tacgatatcc aaaaacattc accgagagca 540
ttgaatgtgg ctgatttgtt aattttgatg gatatctggc caccaggggt actgcccattg 600
tttcaaacca tcgcacctgc cagttctcta acatggcacc tcacttttgt tcgtcccgtt 660
gcttatgagt tacatgattg gtttaagtat caggtcgca cgcagcatgc tgcctttggc 720
tatgctacag aatatgcca tttatgggat gctcaaaacc gtctgattgc tatttcgagg 780
cagacagtta ctgtttttgc ctag 804

```

```

<210> SEQ ID NO 14
<211> LENGTH: 789
<212> TYPE: DNA
<213> ORGANISM: Caulobacter crescentus CB15
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of hypothetical protein
CC2472
(GeneID:943632) (figure 24)

```

<400> SEQUENCE: 14

```

atgacgctct acaccgacct cgtcggggcc atcgctcga cggaaactgg cttttccgcc 60
catgtctccg acgactggaa gcaaggccgc accacctatg gcggcttgag cggcgccttg 120
tgcgtcgagg ccgccttgcg agcctttccc gagggccctc ctctgegetc ggcgcaattc 180
gcctttgtcg gcccgccggc cggcgagttg gcgatctcgg tgcggccgct gcggcagggc 240
aagtcgacgc ttttctgctc cgtcgatctg atcggcgaac agggcgtggc caccacggc 300
gtgctgacct tcggcgccgc gcggacctca gccatctcgt acgaagaagt cctctgcccg 360
cccgtggcgc cggccggcgc ctgtgagctg ttcttccccg aaaatcgcca gggcgcgccc 420
cacttctcgg cgcagttcga ggtgcgcaag gccggcggca cgcgccccct ggccgggggt 480
gagccggaat atctgctgtg gatccgccac cgcgatccgg ccgcgacctc gatctcggcc 540
ctgggtggcgt tggccgacat gccgcccgcc ccggccatgg cgtgttccc gcagtttggg 600
ccgatctcga ccatgacctg gtcgctggat atcgtgggcc tgcccaggc ggacgacgac 660
ggctggcggc tgctgcgcac ccgggcccag accatcggcg acggctactc gaccagggag 720
atgcatctgt gggacgcaa gggccgcccg ctggtcctgg cgcgacagaa cgtggcgatc 780
ttcgtctga 789

```

```

<210> SEQ ID NO 15
<211> LENGTH: 777
<212> TYPE: DNA
<213> ORGANISM: Erythrobacter litoralis HTCC2594
<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of hypothetical protein
ELI0992 (locus_tag=ELI0992) (figure 25)

```

<400> SEQUENCE: 15

```

atgtccgttt ccgatcttcg tgcgccgatc acgtccgaag gtggcgctgt cactcttcca 60
gccgacaaat ggctgcaggc ccgcacgctc tttggcggag cctcggcgct tgcgcctac 120

```

-continued

```

accgccgcccg tgcgcgcttt ccccgatctc ccgcccttgc gcgcagcgca gatcggattt 180
gtcgcgcccgg tcggaaagga cgtggagacg cgggccgcaa tggtcgaca gggtcgcaac 240
gtcgcgcagg tgcgcagcga actgctggtc gaaggcaagg tgcgcgtcac cgcattctgg 300
ctgttcggaa ccggtcgcga ggccaacgcc gtacatgccg ctgccaaggc cgatccctgg 360
cccggcgcac cggaagagaa cgattccgcg atgaccgaca agggcccgcc tttcatcgtc 420
aacaatttcg acattcgccg cgcgcaggaa acgcaaggcc ccggcgaacc gatcgtccgg 480
cgctggttca ggctgaccga tcggggcgag ctcgatcgcg taccggagct gatcctgggtg 540
ggcgatacgc tgccccggg cgcctatgccc gcgatgcagc gccagggccc gatcagctcg 600
atcaactggt cgttcaatat tctcgatgcg gaactcggca cgcgcgacgg ctgggtggctc 660
ggcgagaccg ccagccagca tgccggtgca ggctattcga gcgagcggct acggctctgg 720
aatgccgacg gcgtgcaggt gatggacgga ttgcaatccg ttgcccgttt cggtga 777

```

<210> SEQ ID NO 16

<211> LENGTH: 265

<212> TYPE: PRT

<213> ORGANISM: Pseudomonas putida KT2440

<220> FEATURE:

<223> OTHER INFORMATION: Amino acid sequence of putative acyl-CoA thioesterase II (protein_id="NP_744457.1") (figure 26)

<400> SEQUENCE: 16

```

Met Thr Phe Asn Gln Leu Leu Asp Ala Val Arg Ala Asn Pro Asp Ser
1          5          10          15
Val Ser Ile Pro Pro Ser Trp Ala Gln Gly Arg Ala Ala Phe Gly Gly
20          25          30
Leu Met Ala Ala Met Val Tyr Glu Thr Met Arg Leu Lys Ile Ser Asp
35          40          45
Asn Arg Pro Val Arg Ser Leu Ala Ile Ser Phe Val Ala Pro Ala Ala
50          55          60
Ala Asp Val Pro Ile Arg Phe Glu Val Glu Val Leu Arg Glu Gly Lys
65          70          75          80
Ala Val Ser Thr Leu Leu Gly Arg Ala Val Gln Asp Gly Gln Val Val
85          90          95
Thr Leu Val Gln Gly Asn Phe Gly Ala Gly Arg Pro Ser Val Val Glu
100         105         110
Val Pro Ala Leu Pro Ala Ile Glu Met Pro Ala Leu Asp Glu Ala Ala
115         120         125
Pro Glu Leu Pro Tyr Ile Lys Gly Val Thr Pro Glu Phe Met Arg His
130         135         140
Val Ala Leu Arg Trp Ala Val Gly Gly Leu Pro Phe Ser Gly Asn Gln
145         150         155         160
Ser Arg Lys Met Gly Gly Trp Val Arg Leu Arg Asp Val Val Glu Glu
165         170         175
Gln Val Asn Glu Ala His Leu Leu Ala Leu Val Asp Ala Trp Pro Pro
180         185         190
Ser Leu Met Pro Phe Leu Lys Gln Pro Ala Ala Gly Ser Thr Leu Thr
195         200         205
Trp Thr Ile Glu Phe Ile Gln Pro Thr Ala Lys Leu Ser Thr Leu Asp
210         215         220
Trp Cys Arg Tyr Cys Val Glu Thr Glu His Ala Arg Asp Gly Tyr Gly

```


-continued

225	230	235	240
His Ala Ala Ala Ala Leu Trp Thr Ala Gln Gly Glu Leu Leu Ala Leu	245	250	255
Ser Arg Gln Thr Val Thr Val Phe Ala	260	265	

<210> SEQ ID NO 17
 <211> LENGTH: 265
 <212> TYPE: PRT
 <213> ORGANISM: Pseudomonas aeruginosa PA01
 <220> FEATURE:
 <223> OTHER INFORMATION: Amino acid sequence of hypothetical protein
 PA2871
 (protein_id="NP_251561.1) (figure 27)

<400> SEQUENCE: 17

Met Asn Phe Ser Glu Leu Ile Gln Ala Val Arg Arg Asp Pro Ser Ser	5	10	15
1			
Val Val Val Pro Ala Ser Trp Gly Gln Gly Arg Ala Thr Phe Gly Gly	20	25	30
Leu Val Val Ala Leu Ala Tyr Glu Ala Met Leu Ala Val Val Glu Ala	35	40	45
Gly Arg Pro Leu Arg Ser Ile Gly Val Ser Phe Val Gly Pro Leu Ala	50	55	60
Pro Glu Gln Pro Ala Ser Phe Ser Ala Arg Leu Leu Arg Glu Gly Lys	65	70	75
			80
Ala Val Ser Gln Val Gln Val Glu Val Arg Gln Gly Glu Gln Val Val	85	90	95
Thr Leu Val Gln Ala Ser Phe Gly Val Ala Arg Ala Ser Ala Val Ala	100	105	110
Val Glu Ala Leu Pro Ala Ala Gly Met Lys Gly Pro Glu Glu Ser Gln	115	120	125
Glu Leu Pro Tyr Ile Arg Asn Val Thr Pro Glu Phe Thr Arg Tyr Ile	130	135	140
Ala Met Arg Trp Ala Val Gly Gly Leu Pro Phe Ser Ser Asn Lys Ser	145	150	155
			160
Arg Gln Met Gly Gly Trp Met Arg Phe Arg Asp Glu Pro Glu Gly Glu	165	170	175
Pro Met Glu Val Ser His Leu Leu Ala Leu Leu Asp Ser Trp Pro Pro	180	185	190
Ala Leu Leu Pro His Leu Gly Thr Pro Ala Met Ala Ser Ser Leu Thr	195	200	205
Trp Thr Ala Glu Phe Leu Gln Pro Leu Pro Gln Gln Gly Ser Gly Asp	210	215	220
Trp Cys Arg Tyr Leu Ala Glu Ile Glu Glu Ala Arg Asp Gly Tyr Gly	225	230	235
			240
His Val Ala Ala Arg Met Trp Ser Ala Asp Gly Gln Leu Leu Ala Ile	245	250	255
Ser Arg Gln Met Val Thr Val Phe Gly	260	265	

<210> SEQ ID NO 18
 <211> LENGTH: 265
 <212> TYPE: PRT
 <213> ORGANISM: Pseudomonas syringae pv B728a
 <220> FEATURE:

-continued

<223> OTHER INFORMATION: Amino acid sequence of acyl-CoA thioesterase II, putative of *p. syringae* pv B728a (protein_id="YP_235138.1") (figure 28)

<400> SEQUENCE: 18

```

Met Thr Phe Ser Glu Leu Ile Asp Ala Leu Arg Arg Asp Pro Arg Ser
1           5           10           15

Val Thr Ile Pro Ala Glu Trp Ser Gln Gly Arg Ala Cys Phe Gly Gly
          20           25           30

Leu Met Ala Ala Leu Thr Tyr Glu Ala Met Arg Ala Gln Val Pro Glu
          35           40           45

Gly Arg Pro Val Arg Ser Leu Ala Ile Thr Phe Val Gly Pro Ala Ala
          50           55           60

Pro Gly Val Pro Ile Ala Phe Glu Val Asp Thr Leu Arg His Gly Lys
65           70           75           80

Ala Val Ser Gln Val Leu Gly Arg Ala Met Gln Asn Gly Gln Val Met
          85           90           95

Thr Leu Ile Gln Gly Ser Phe Gly Ala Pro Arg Glu Ser Met Ile Thr
          100          105          110

Val Ala Ala Glu Ala Ala Pro Val Leu Lys Pro Val Asp Gln Cys Pro
          115          120          125

Glu Leu Pro Phe Ala Ser Gly Val Met Pro Asp Tyr Leu Arg Phe Met
130          135          140

Asp Ile Arg Trp Ala Leu Gly Gly Met Pro Phe Ser Asn Thr Arg Ser
145          150          155          160

Pro Ala Ile Gly Gly Tyr Val Arg Phe Arg Asp Thr Pro His Ala Thr
          165          170          175

Pro Met Ser Glu Ala His Ile Leu Ala Leu Val Asp Thr Trp Pro Pro
          180          185          190

Ala Val Leu Pro His Leu Asp Lys Pro Ala Pro Gly Ser Ser Leu Thr
          195          200          205

Trp Thr Ile Glu Phe Val Gln Pro Gln Pro Ser Leu Asp Thr Leu Gln
210          215          220

Trp Cys Ser Tyr Arg Ala Val Ile Glu His Ala Arg Asp Gly Tyr Gly
225          230          235          240

His Thr Ala Ala Ala Leu Trp Ser Pro Asp Gly Glu Leu Ile Ala Ile
          245          250          255

Ser Arg Gln Thr Val Thr Val Phe Gly
          260          265

```

<210> SEQ ID NO 19

<211> LENGTH: 264

<212> TYPE: PRT

<213> ORGANISM: *Pseudomonas fluorescens* Pf0-1

<220> FEATURE:

<223> OTHER INFORMATION: Amino acid sequence of acyl-CoA thioesterase (protein_id="ZP_00264181.1") (figure 29)

<400> SEQUENCE: 19

```

Met Arg Phe Cys Asp Leu Ile Asp Ala Val Arg Arg Gln Pro Glu Val
1           5           10           15

Thr Ile Pro Ala Glu Trp Gly Gln Gly Arg Ala Ser Phe Gly Gly Leu
          20           25           30

Val Ala Ala Leu Gln Phe Glu Val Met Arg Thr Lys Val Pro Thr Asp
          35           40           45

```

-continued

Arg Pro Val Arg Ser Leu Ala Ile Thr Phe Val Gly Pro Val Glu Pro
 50 55 60
 Glu Val Pro Val Ser Phe Glu Val Glu Val Leu Arg Glu Gly Lys Ala
 65 70 75 80
 Val Ser Gln Val Leu Gly Arg Ala Val Gln Asn Gly Gln Val Val Thr
 85 90 95
 Met Val Gln Gly Ser Phe Gly Ala Ser Arg Pro Ser Glu Val Ala Val
 100 105 110
 Glu Ala Tyr Pro Ala Pro Glu Met Lys His Trp Asp Asp Cys Gln Glu
 115 120 125
 Leu Pro Tyr Ile Lys Gly Val Thr Pro Glu Phe Met Arg His Leu Ala
 130 135 140
 Met Arg Trp Ser Val Gly Gly Met Pro Phe Thr Gly Asn Gln Ser Arg
 145 150 155 160
 Leu Met Gly Gly Trp Val Arg Leu Arg Gly Asp Val Lys Glu Glu Ser
 165 170 175
 Val Asn Glu Ala His Leu Leu Ala Leu Val Asp Ala Trp Pro Pro Ala
 180 185 190
 Leu Leu Pro Tyr Leu Lys Lys Pro Ala Pro Gly Ser Thr Leu Thr Trp
 195 200 205
 Thr Ile Glu Phe Val Gln Pro Leu Arg Asp Leu Ser Thr Leu Asp Phe
 210 215 220
 Cys Gln Tyr Leu Ala Asp Ile Glu Tyr Ala Ala Asp Gly Tyr Gly His
 225 230 235 240
 Val Ala Ala Lys Leu Trp Ser Ala Lys Gly Glu Leu Ile Ala Met Ser
 245 250 255
 Arg Gln Thr Val Thr Ile Phe Ala
 260

<210> SEQ ID NO 20
 <211> LENGTH: 264
 <212> TYPE: PRT
 <213> ORGANISM: *Idiomarina loihiensis* L2TR
 <220> FEATURE:
 <223> OTHER INFORMATION: Amino acid sequence of tesB-like acyl-CoA
 thioesterase (protein_id=AAV81497.1) (figure 30)

<400> SEQUENCE: 20

Met Asn Phe His Thr Ala Val Glu Gln Ile Val Ala Asp Lys Asn Asn
 1 5 10 15
 Gln Val Ile Glu Leu Pro Ser Gly Trp Ala Gln Gly Arg Ala Phe Phe
 20 25 30
 Gly Gly Phe Ser Gly Ala Leu Ala Ala Gln Phe Leu Leu Lys Gln Phe
 35 40 45
 Pro Ile Glu Tyr His Leu Arg Ser Met Ser Ile Ser Phe Val Ala Pro
 50 55 60
 Ala Glu Pro Gly Glu Ala Glu Leu Asn Tyr Arg Ile Leu Arg Glu Gly
 65 70 75 80
 Lys Ser Val Ile Gln Val Ala Val Glu Leu Gln Gln Gln Gly Gln Ile
 85 90 95
 Met Leu Ser Cys Leu Ala Ser Leu Gly Lys Gly Arg Ser Ser Thr Val
 100 105 110
 Thr Val Val Ser Glu Thr Pro Pro Asp Leu Lys Thr Ile Asn Asp Gly
 115 120 125

-continued

Pro Gly Leu Pro Glu Ala Asp Ile Val Pro Glu Phe Ala Lys Asn Phe
 130 135 140

Asp Tyr Arg Ile Thr Ser Gly Gly Met Pro Phe Ser Gly Gln Pro Gly
 145 150 155 160

Arg Thr Phe Gly Gly Trp Ile Arg Phe Arg Glu Glu Gln Gln Pro Leu
 165 170 175

Thr Thr Ala Thr Ile Leu Ala Leu Val Asp Ala Trp Pro Pro Ala Val
 180 185 190

Leu Pro His Leu Asp Ser Pro Ala Pro Ala Ser Ser Leu Thr Trp Thr
 195 200 205

Ile Glu Phe Pro Asp Ile Pro Leu Gln Ser Phe Ser Ser His Asp Trp
 210 215 220

Phe Gln Tyr Glu Ala Phe Ile Glu His Ala Glu Asn Gly Tyr Gly His
 225 230 235 240

Ser Arg Ala Gly Leu Trp Ser Glu Lys Gly Glu Leu Leu Ala Ile Ser
 245 250 255

Arg Gln Thr Phe Thr Val Phe Ala
 260

<210> SEQ ID NO 21
 <211> LENGTH: 267
 <212> TYPE: PRT
 <213> ORGANISM: Acinetobacter sp. ADP1
 <220> FEATURE:
 <223> OTHER INFORMATION: Amino acid sequence of putative acyl-CoA
 thioesterase II (protein_id=CAG67294.1) (figure
 31)

<400> SEQUENCE: 21

Met Ser Asp Ala Met Pro Leu Asp Gln Thr Leu Glu Gln Leu Thr Gln
 1 5 10 15

Asp Glu Trp Ile Asp Ile Pro Gln Gly Trp Ser Gln Gly Arg Thr Ile
 20 25 30

Tyr Gly Gly Leu Val Ala Gly Leu Leu Met His Lys Ala Leu Ser Val
 35 40 45

Met Asn Asp Glu Ser Lys Asn Leu Leu Ser Thr Ser Ile Thr Phe Val
 50 55 60

Gly Pro Val Asn Glu Gly Arg Val Arg Leu Thr Val Glu Ile Leu Arg
 65 70 75 80

Gln Gly Lys Ser Val Thr Thr Ile Glu Ala Arg Leu Trp Gln Asp Gln
 85 90 95

Ala Val Gln Thr Ile Leu Ile Ala Ser Phe Gly Gln Pro Arg Ser Ser
 100 105 110

Glu Ile Phe Val Leu Asn Leu Pro Glu Ala Pro Asp Tyr Leu Ser Pro
 115 120 125

Glu Gln Phe Ser Arg Met Pro Phe Val Lys Met Met Pro Glu Cys Tyr
 130 135 140

Gln Gln Phe Asp Leu Arg Trp Ala Glu Gly His Tyr Pro Met Thr Gln
 145 150 155 160

Gln Ala Pro Asp Phe Gly Gly Trp Cys Arg Tyr Asp Ile Gln Lys His
 165 170 175

Ser Pro Arg Ala Leu Asn Val Ala Asp Leu Leu Ile Leu Met Asp Ile
 180 185 190

Trp Pro Pro Gly Val Leu Pro Met Phe Gln Thr Ile Ala Pro Ala Ser

-continued

<210> SEQ ID NO 23
 <211> LENGTH: 258
 <212> TYPE: PRT
 <213> ORGANISM: *Erythrobacter litoralis* HTCC2594
 <220> FEATURE:
 <223> OTHER INFORMATION: Amino acid sequence of hypothetical protein
 ELI0992 (protein_id=ZP_00375750.1) (figure 33)

<400> SEQUENCE: 23

Met Ser Val Ser Asp Leu Arg Ala Pro Ile Thr Ser Glu Gly Gly Ala
 1 5 10 15
 Val Thr Leu Pro Ala Asp Lys Trp Leu Gln Gly Arg Thr Leu Phe Gly
 20 25 30
 Gly Ala Ser Ala Leu Val Ala Tyr Thr Ala Ala Val Arg Ala Phe Pro
 35 40 45
 Asp Leu Pro Pro Leu Arg Ala Ala Gln Ile Gly Phe Val Ala Pro Val
 50 55 60
 Gly Lys Asp Val Glu Thr Arg Ala Ala Met Val Arg Gln Gly Arg Asn
 65 70 75 80
 Val Ala Gln Val Arg Ser Glu Leu Leu Val Glu Gly Lys Val Ala Leu
 85 90 95
 Thr Ala Phe Trp Leu Phe Gly Thr Gly Arg Glu Ala Asn Ala Val His
 100 105 110
 Ala Ala Ala Lys Ala Asp Pro Trp Pro Gly Ala Pro Glu Glu Asn Asp
 115 120 125
 Ser Ala Met Thr Asp Lys Gly Pro Pro Phe Ile Val Asn Asn Phe Asp
 130 135 140
 Ile Arg Arg Ala Gln Glu Thr Gln Gly Pro Gly Glu Pro Ile Val Arg
 145 150 155 160
 Arg Trp Phe Arg Leu Thr Asp Arg Gly Glu Leu Asp Arg Val Ser Glu
 165 170 175
 Leu Ile Leu Val Gly Asp Thr Leu Pro Pro Gly Ala Met Arg Ala Met
 180 185 190
 Gln Arg Gln Gly Pro Ile Ser Ser Ile Asn Trp Ser Phe Asn Ile Leu
 195 200 205
 Asp Ala Glu Leu Gly Thr Arg Asp Gly Trp Trp Leu Gly Glu Thr Ala
 210 215 220
 Ser Gln His Ala Gly Ala Gly Tyr Ser Ser Glu Arg Leu Arg Leu Trp
 225 230 235 240
 Asn Ala Asp Gly Val Gln Val Met Asp Gly Leu Gln Ser Val Ala Val
 245 250 255
 Phe Gly

<210> SEQ ID NO 24
 <211> LENGTH: 25
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence:
 Primer (OTR
 End) used in Example 1

<400> SEQUENCE: 24

ggccgcactt gtgtataaga gtcag

25

-continued

<210> SEQ ID NO 25
 <211> LENGTH: 25
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence:
 Primer (1TR
 End) used in Example 1

 <400> SEQUENCE: 25

 gcgccagat ctgatcaaga gacag 25

<210> SEQ ID NO 26
 <211> LENGTH: 21
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Primer
 1086F
 used in Example 1

 <400> SEQUENCE: 26

 ttactggctt cgcaggaatg g 21

<210> SEQ ID NO 27
 <211> LENGTH: 26
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Primer
 IntSM160 used in Example 1

 <400> SEQUENCE: 27

 cttggcacc agcatgcgcg agcagg 26

<210> SEQ ID NO 28
 <211> LENGTH: 21
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Primer
 1087F
 used in Example 3

 <400> SEQUENCE: 28

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 <223> OTHER INFORMATION: Description of Artificial Sequence: Primer
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 used in Example 3

 <400> SEQUENCE: 29

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<210> SEQ ID NO 30
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 <223> OTHER INFORMATION: Nucleotide sequences of the genes tesB-like
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 and plsC (Abo_1045) consisting of an operon in

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Alcanivorax borkumensis, with Tn5 inserted at position 588.

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<210> SEQ ID NO 31
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Nucleotide sequence of tesB-like acyl-CoA
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<400> SEQUENCE: 31

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1. A genetically engineered microorganism having at least one modification in at least one gene encoding a protein involved in the metabolism of polyhydroxyalkanoate(s) (PHA), or in at least one gene encoding a protein, which interferes in the metabolism of the microorganism with the production of PHA, wherein the at least one modification causes deposition and/or overproduction of medium or long chain polyhydroxyalkanoate(s) PHA.

2. The genetically engineered microorganism of claim 1, wherein the at least one gene encodes a protein, which interferes in the metabolism of the microorganism with the production of PHA, wherein the protein is an enzyme, which competes with enzymes of the PHA synthesis pathway for intermediates of the PHA synthesis pathway.

3. The genetically engineered microorganism of claim 2, wherein the enzyme is a thioesterase.

4. The genetically engineered microorganism of claim 2, wherein the thioesterase acts on (R)-3-OH-acyl-CoA as a substrate.

5. The microorganism of claim 2, wherein the thioesterase is acyl-CoA thioesterase tesB-like.

6. The genetically engineered microorganism of claim 2, wherein the enzyme is encoded by a nucleic acid sequence comprising a (modified) nucleic acid sequence as shown in any of FIGS. 11 and 12, or a homolog thereof.

7. The genetically engineered microorganism of claim 2, wherein the enzyme is encoded by a nucleic acid sequence comprising a (native) nucleic acid sequence as shown in any of FIGS. 14, 16 and 18 to 25, or a homolog thereof, wherein the native nucleic acid sequence is modified by the at least one modification.

8. The genetically engineered microorganism of claim 1, wherein the at least one modification is causes a complete or partial inactivation of the modified gene.

9. The genetically engineered microorganism of claim 1, wherein the at least one modification is effected by a transposon insertion selected from the group consisting of Tn5 and Tn10 transposons, preferably by a Tn5 insertion, more preferably on the gene downstream to the modified gene.

10. The genetically engineered microorganism of claim 1, wherein the at least one modification is effected by transpo-

son mutagenesis, preferably based on miniTn5 Km element, more preferably based on miniTn5 Str/Sp element.

11. The genetically engineered microorganism of claim 1, wherein the gene is integrated into the chromosome of the microorganism.

12. The genetically engineered microorganism of claim 1, wherein the at least one gene encodes a protein selected from the group consisting of PHA synthase, PHB synthase, acyl-CoA transferase, enoyl-CoA hydratase or reductase.

13. The genetically engineered microorganism of claim 1, wherein the at least one modification causes an extracellular deposition or an overproduction of polyhydroxyalkanoate(s) PHA, preferably medium or long chain polyhydroxyalkanoates (PHA), produced by the microorganism.

14. The genetically engineered microorganism of claim 13, wherein the extracellular deposition or overproduction of polyhydroxyalkanoate(s) PHA, results in a PHA production which is at least 5 times, preferably at least 10 times, more preferably at least 15 times, more preferably at least 25 times, even more preferably at least 40 times, even more preferably at least 50 times, most preferably at least 60 times, most preferably at least 80 times, even most preferably at least 100 times higher than the PHA production of the corresponding wild type microorganism.

15. The genetically engineered microorganism of claim 1, wherein the microorganism is selected from the group consisting of *Alcanivorax borkumensis*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Pseudomonas acitophila*, *Pseudomonas oleovarans*, *Idiomarina loihiensis*, *Acinetobacter sp.*, *Caulobacter crescentus*, *Alcaligenes eutrophus*, *Alcaligenes latus*, *Azotobacter vinlandii*, *Rhodococcus eutropha*, *Chromobacterium violaceum*, and *Chromatium vinosum*.

16. The genetically engineered microorganism of claim 1, wherein the microorganism is selected from the group consisting of *Alcanivorax*, preferably *Alcanivorax borkumensis*, more preferably *Alcanivorax borkumensis* SK2.

17. A nucleic acid sequence which constitutes at least one gene encoding a protein involved in the metabolism of polyhydroxyalkanoate(s) (PHA), or in at least one gene encoding a protein, which interferes in the metabolism of the microor-

ganism with the production of PHA, wherein the at least one modification causes deposition and/or overproduction of medium or long chain polyhydroxyalkanoate(s) PHA.

18. The nucleic acid sequence of claim **17**, wherein the gene is selected from the group consisting of PHA synthase, PHB synthase, acyl-CoA transferase, and enyol-CoA.

19. The nucleic acid sequence of claim **17** wherein the gene is acyl-CoA thioesterase tesB-like, preferably acyl-CoA thioesterase tesB-like of *Alcanivorax borkumensis*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Pseudomonas acitophila*, *Pseudomonas oleovorans*, *Idiomarina loihiensis*, *Acinetobacter sp.*, *Caulobacter crescentus*, *Alcaligenes eutrophus*, *Alcaligenes latus*, *Azotobacter vinlandii*, *Rhodococcus eutropha*, *Chromobacterium violaceum*, or *Chromatium vinosum*.

20. The nucleic acid sequence of claim **17** wherein the gene is acyl-CoA thioesterase tesB-like, preferably acyl-CoA thioesterase tesB-like of *Alcanivorax borkumensis*, more preferably acyl-CoA thioesterase tesB-like of *Alcanivorax borkumensis* SK2.

21. The nucleic acid sequence of claim **17**, wherein the at least one modification is effected by a transposon insertion, preferably Tn-5 insertion, on the gene downstream to the modified gene.

22. The nucleic acid sequence of claim **17**, wherein the at least one modification is achieved by transposon mutagenesis, preferably based on miniTn5 Km element, more preferably based on miniTn5 Str/Sp element.

23. The nucleic acid sequence of claim **17**, wherein the nucleic acid comprises a (modified) nucleic acid sequence as shown in any of FIGS. **11** and **12**, or a homolog thereof.

24. The nucleic acid sequence of claim **17**, wherein the nucleic acid comprises a (native) nucleic acid sequence as shown in any of FIGS. **14**, **16** and **18** to **25**, or a homolog thereof, wherein the native nucleic acid sequence is modified by the at least one modification.

25. Polypeptide encoded by a nucleic acid sequence of claim **17**.

26. Polypeptide of claim **25**, wherein the polypeptide comprises one of the amino acid sequences shown in FIG. **11** or **12**.

27. A vector comprising the nucleic acid sequence of claim **17**.

28. A vector comprising a PHA producing gene cluster comprising a gene having at least one modification in at least one gene encoding for a protein involved in the metabolism of polyhydroxyalkanoate(s) (PHA), or in at least one gene encoding for a protein, which interferes in the metabolism of the genetically engineered microorganism of claim **1** with the production of PHA, wherein the at least one modification causes extracellular deposition of medium or long chain polyhydroxyalkanoate(s) (PHA).

29. A cell comprising the vector of claim **27** and/or the nucleic acid sequence of claim **17**.

30. A method for producing polyhydroxyalkanoates (PHA) comprising the following steps:

a. cultivating a microorganism of claim **1** or a cell of claim **29** and

b. recovering polyhydroxyalkanoates (PHA) from the culture medium.

31. (canceled)

32. (canceled)

* * * * *