Extracellular polyhydroxyalkanoates produced by genetically engineered microorganisms

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).

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.

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 Roukhelman, 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 (Steinbuchel, 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; US Patent No. 4,876,331). These copolymers can be referred to as PHB-co-HX (wherein X is a 3-hydroxyalkanoate or alkanoate 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 & McInerey, 1991, Arch. Microbiol. 155: 103-06; US Patent No. 5,292,860).

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 oleovarans, Eschericha coli, Rhodococcus eutropha, Chromobacterium violaceum, Chromatium vinosum, Alcanivorax borkumensis* etc.. All PHA producing bacteria known in the art produce intracellular PHA and accumulate it in PHA granules (Steinbüchel, 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

i) an appropriate solvent,

ii) hypochlorite extraction of PHA and/or

iii) digestion of non-PHA cellular materials.

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.

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.

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.

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 Figures 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 Figures 11, 12, 14, 16 und 18 to 25, or a functional fragment or variant thereof.

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".

Various genes encoding polypeptides are involved in the metabolism of PHA. Several of these genes are specified in Table 1 as represented by Figure 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, enyol-CoA hydratase, and PHB synthase.

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.

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.

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, WM *et al.*, 1972, J. Biol. Chem. 247, 3123-3133), while thioesterase II cleaves C6 to C18 acyl-CoA esters as well as ß-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 ß-oxidation in *E. coli* (Klinke, SQ *et al.*, 1999, Appl. Environ. Microbiol. 65: 540-548). Up to now, little was known on physiological role of *tesB* in bacterial metabolism.

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 Figure 8).

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.

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.

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.

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)).

Both types of thioesterases being present in *Alcanivorax 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 Figure 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, RA *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).

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.

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 Fig 11 to 14 and 15 to33 as well as Examples).

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.

Additionally, modifications in one or more of the genes involved in the PHA synthesis may occur in the microorganism according to the invention. These modifications may be directed to PHA synthase, poly(3-hydroxyalkanoate)synthase, enyol-CoA hydratase,

and/or PHB synthase and/or other enzymes involved in the fatty acid metabolism. Genes encoding for enoyl-CoA hydratase in Alcanivorax 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 Alcanivorax: 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.

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).

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).

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 introduced 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:

1. isolation and fragmentation of the source DNA (e.g. genomic DNA, cDNA, synthetic DNA etc.)
2. joining DNA fragments to a cloning vector with DNA ligase and
3. introduction and maintenance in a host organism (microorganism), e.g., by transformation.

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 my be incorporated into the chromosome of the cell.

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 f1 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 oliognucleotide, 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.

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 Figures 14 and 15), more preferably miniTn5 streptomycin element (miniTn Str/Sp element) (see Example 1).

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.

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.

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 extracelluar 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".

According to the invention, overproduction of PHA by the microorganism of the invention has been shown (see Figures 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.

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, enyol-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).

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 all. 1998). However, culturing conditions were not favorable for PHA accumulation due to relatively high nitrogen concentration in the culture medium (5g/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*.

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, enyol-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 (Figure 10).

An especially preferred microorganism of the present invention has been deposited according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1b, 38124 Braunschweig, Germany with the identification reference SK2 C9 mutant and the Accession Number DSM17483.

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

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.

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.

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 Figures 16 and 18 to 25 or encode native polypeptides sequences according to Figures 17 and 26 to 33, wherein these sequences have been interrupted by transposon inserts (e.g. as exemplified in Fig. 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 Figures 11 and 12, which have been interrupted by transposon inserts.

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.

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 complementary 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.

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.

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.

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.

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.

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 Figures 13, 17 and 26 to 33.

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 Habor, NY) and are also described by this invention, e.g. Examples 3 to 8, Figures 1, 3, 4 to 6). Without being limited thereto, "functional" polypeptides according to the invention may comprises native polypeptides sequences according to Figures 17 and 26 to 33.

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 reaction 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 Figures 17 and

26 to 33 or as encoded by nucleic acid sequences according to Figures 16 and 18 to 25, wherein these sequences have been interrupted by transposon inserts (e.g. as exemplified in Fig. 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 Figures 11 and 12, which have been interrupted by transposon inserts.

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*).

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.

The invention also provides a vector comprising the nucleic acid sequence of the invention. The terms "construct", "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.

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, colEl 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.

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.

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.

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 promotor) 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, CA.

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

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*.

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.

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 tranduction) 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.

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 or this vector occurs within the cell.

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

- cultivating a microorganism or a cell of the invention and
- recovering polyhydroxyalkanoates (PHA) from the culture medium.

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*.

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).

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).

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).

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 microorganism 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.

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.

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.

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

Figure 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 deposites, e.g. excretes, and overproduces PHA.

Figure 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 NH_4Cl (storage conditions) and harvested in stationary phase of growth. These ultrathin sections prepared from the cells confirmed presence of intracellular granula.

Figure 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.

Figure 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.

Figure 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.

Figure 6 shows **Table 2** representing the analysis of PHA and its composition.

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.

The amount of Pha isolated from C9 mutant cells grown on either pyruvate or octadecane was too low for quantification (see also Figure 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).

As shown in Table 2, the amount of PHA produced by SK2 wild type was rather low (6.5 mg/l on pyruvate and 18mg/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. borkumenis* SK2 under high C/N ratio indeed showed granula visible under light microscope consisting of PHA (see also Fig. 2).

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.

Figure 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.

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

Figure 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.* 1999 Hypothetical pathway of MCL PHA biosynthesis of PHA polymerase- and thioesterase I-containing *E. coli* JMU193 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 b-oxidation in form of acyl-CoA. The (S)-3-OH-acyl-CoA produced in beta 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 *tes B*. Tree 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 snthase. The mutation in the *tesB-like* acyl-CoA thioesterase abolishes production of 3-HAA and results in uncontrolled production of PHA.

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.

Figure 9 shows the operon structure of the two genes *tesB-like* and „putative acetyltransferase“ forming a single operon.

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 Figure 9.

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.

Figure 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.* ADP1 and *Caulobacter crescentus* CB15. 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).

Figure 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).

Figure 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.

Figure 13 shows the amino acid sequence of *tesB-like* of *A.borkumensis*.

Figure 14 shows the nucleic acid sequence of miniTn5 Km element.

Figure 15 shows the amino acid sequence of neomycin phosphotransferase responsible for neomycin and kanamycin resistance.

Figure 16 shows the nucleic acid sequence of „putative acetyltransferase" of *A.borkumensis*. Start and stop codons are marked in bold letters.

Figure 17 shows the amino acid sequence of „putative acetyltransferase" of *A.borkumensis*.

Figure 18 shows the nucleic acid sequence of putative acyl-CoA thiosterase II of *Pseudomonas putida* KT2440.

Figure 19 shows the nucleic acid sequence of hypothetical protein PA2871 of *Pseudomonas aeruginosa* PA01.

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

Figure 21 shows the nucleic acid sequence of acyl-CoA thiosterase of *Pseudomonas fluorescens* PfO-1.

Figure 22 shows the nucleic acid sequence of *tesB-like* acyl-CoA thiosterase of *Idiomarina loihiensis* L2TR.

Figure 23 shows the nucleic acid sequence of putative acyl-CoA thioesterase II of *Acinetobacter sp.* ADP1.

Figure 24 shows the nucleic acid sequence of hypothetical protein CC2472 of *Caulobacter crescentus* CB15.

Figure 25 shows the nucleic acid sequence of hypothetical protein ELI0992 of *Erythrobacter litoralis* HTCC2594.

Figure 26 shows the amino acid sequence of putative acyl-CoA thiosterase II of *Pseudomonas putida* KT2440.

Figure 27 shows the amino acid sequence of hypothetical protein PA2871 of *Pseudomonas aeruginosa* PA01.

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

Figure 29 shows the amino acid sequence of acyl-CoA thiosterase of *Pseudomonas fluorescens* PfO-1.

Figure 30 shows the amino acid sequence of *tesB-like* acyl-CoA thiosterase of *Idiomarina loihiensis* L2TR.

Figure 31 shows the amino acid sequence of putative acyl-CoA thioesterase II of *Acinetobacter sp.* ADP1.

Figure 32 shows the amino acid sequence of hypothetical protein CC2472 of *Caulobacter crescentus* CB15.

Figure 33 shows the amino acid sequence of hypothetical protein ELI0992 of *Erythrobacter litoralis* HTCC2594.

Examples

Materials: Bacterial strains, media and growth conditions

A.borkumensis SK2 was used as the wild type for all experiments. The bacteria were grown at 30 ^{0}C in modified ONR7a medium (Yakimov et al 1998) where 0.27 g/l of NH_4Cl was used as the source of nitrogen instead of KNO_3. Pyruvate (2%) or octadecane (1.5%) were used as a carbon and energy source.

E.coli was grown at 37 ^{0}C in Luria-Bertani medium supplemented with streptomycin (50µg/ml), chloramphenicol (12,5µg/ml), kanamycin (50µg/ml), nalidixic acid (10µg/ml)

as necessary. Plasmids were introduced into *A.borkumensis* by conjugation with *E.coli* strain S17-1.

Example 1: Mini-Tn5 mutagenesis

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 ^{0}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 ^{0}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 ($Na_2HPO_4.2H_2O$, 0.45 g/l; $NaNO_3$, 2.5 g/l; NaCl, 11.5 g/l; KCl, 0.38 g/l; $CaCl_2.2H_2O$, 0.7 g/l) and 2% pyruvate as carbon and energy source. The plate was incubated for 24 hours at 30^{0}C. The cells were then washed with 10mM $MgSO_4$ and the transconjugants were selected on ONR7a with nalidixic acid and streptomycin.

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 ^{0}C for 1,5 min; 48 ^{0}C for 1 min; 70 ^{0}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 disrupted gene: 1086F (TTA CTG GCT TCG CAG GAA TGG) and IntSM160 (CTT GGC ACC CAG CAT GCG CGA GCA GG9).

Example 2: Biofilm formation assay

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.

Biofilm formation was quantified by the addition of 2 x 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 polysterene microtitre dish, and the adsorbance was determined with a plate reader at 600 nm (series 700 microplate reader; Cambridge Technology).

Example 3: Site-directed mutagenesis of „putative acetyltransferase" gene

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: (CTTTGATCAGTCCGGCAAAAC) 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: Chemical analysis of PHA

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 (100rpm) at 30 ^{0}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: PHA quantification

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: Gas chromatography/mass spectroscopy

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 destilled 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, TX, and VG Autospec spectrometer), equipped with a 30 m x 0.25 mm HP-5 (5% diphenyl and 95% dimethylpolysiloxane) fused silica capillary column; flow rate 1 ml/min; 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: Gel permeation chromatography

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: Electron microscopy

Cells were cultivated in ONR7a containing 1.5% (w/v) octadecane and NH_4Cl (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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Claims

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 any of claims 2 or 3, wherein the thioesterase acts on (R)-3-OH-acyl-CoA as a substrate.

5. The microorganism of any of claims 2 or 3, wherein the thioesterase is acyl-CoA thioesterase *tesB-like*.

6. The genetically engineered microorganism of any of claims 2 to 5, wherein the enzyme is encoded by a nucleic acid sequence comprising a (modified) nucleic acid sequence as shown in any of Figures 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 Figures 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 any of claims 1 to 7, wherein the at least one modification is causes a complete or partial inactivation of the modified gene.

9. The genetically engineered microorganism of any of claims 1 to 8, 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 any of claims 1 to 9, wherein the at least one modification is effected by transposon mutagenesis, preferably based on miniTn5 Km element, more preferably based on miniTn5 Str/Sp element.

11. The genetically engineered microorganism of any of claims 1 to 10, 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, enyol-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 any of claims 1 to 14, 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, Chromatium vinosum.*

16. The genetically engineered microorganism of any of claims 1 to 15, 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 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.

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, 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 oleovarans, Idiomarina loihiensis, Acinetobacter sp., Caulobacter crescentus, Alcaligenes eutrophus, Alcaligenes latus, Azotobacter vinlandii, Rhodococcus eutropha, Chromobacterium violaceum, 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 any of claims 17 to 20, 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 any of claims 17 to 21, 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 any claims 17 to 22, wherein the nucleic acid comprises a (modified) nucleic acid sequence as shown in any of Figures 11 and 12, or a homolog thereof.

24. The nucleic acid sequence of any claims 17 to 22, wherein the nucleic acid comprises a (native) nucleic acid sequence as shown in any of Figures 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 any of claims 17 to 24.

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

27. A vector comprising the nucleic acid sequence of any of claims 17 to 24.

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 any of claims 1 to 16 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 or 28 and/or the nucleic acid sequence of any of claims 17 to 24.

30. A method for producing polyhydroxyalkanoates (PHA) comprising the following steps:
    a. cultivating a microorganism of any of claims 1 to 16 or a cell of claim 29 and
    b. recovering polyhydroxyalkanoates (PHA) from the culture medium.

31. Use of a microorganism of any of claims 1 to 16 and/or the nucleic acid sequence of any of claims 17 to 24 and/or the vector of claim 26 or 27 and/or the cell of claim 29 for the production and deposition of medium and/or long chain polyhydroxyalkanoates (PHA).

32. Use of a microorganism of any of claims 1 to 16 and/or the nucleic acid sequence of any of claims 17 to 24 and/or the vector of claim 26 or 27 and/or the cell of claim 29 for the overproduction of medium and/or long chain polyhydroxyalkanoates (PHA).