Bioplastics from microorganisms

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The term 'biomaterials' includes chemically unrelated products that are synthesised by microorganisms (or part of them) under different environmental conditions. One important family of biomaterials is bioplastics. These are polyesters that are widely distributed in nature and accumulate intracellularly in microorganisms in the form of storage granules, with physico-chemical properties resembling petrochemical plastics. These polymers are usually built from hydroxy-acyl–CoA derivatives via different metabolic pathways. Depending on their microbial origin, bioplastics differ in their monomer composition, macromolecular structure and physical properties. Most of them are biodegradable and biocompatible, which makes them extremely interesting from the biotechnological point of view.

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Abbreviations

lcl	long-chain length
mcl	medium-chain length
PHA	poly(3-hydroxyalkanoate)
PHB	poly(3-hydroxybutyrate)
PM	phospholipid monolayer
scl	short-chain length

Introduction

The exponential growth of the human population has led to the accumulation of huge amounts of non-degradable waste materials across our planet. Living conditions in the biosphere are therefore changing dramatically, in such a way that the presence of non-biodegradable residues is affecting the potential survival of many species. For this reason, many countries have promoted special programmes directed towards the discovery of new commonly used materials that can be readily eliminated from the biosphere, and have designed novel strategies aimed at facilitating the transformation of contaminants.

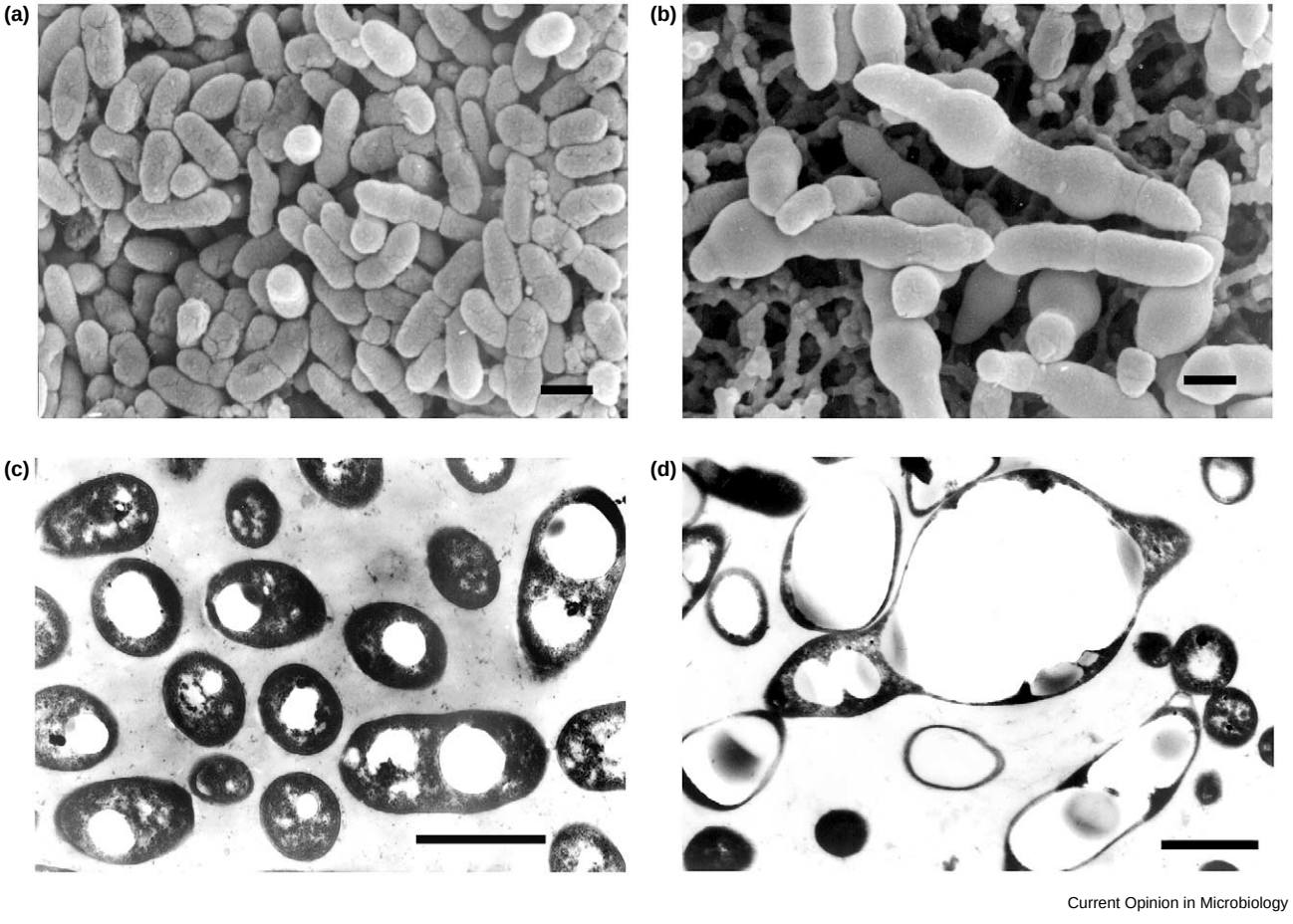
Biomaterials are natural products that are synthesised and catabolised by different organisms and that have found broad biotechnological applications. They can be assimilated by many species (biodegradable) and do not cause toxic effects in the host (biocompatible) [1,2,3•,4••], conferring upon them a considerable advantage with respect to other conventional synthetic products.

Bioplastics are a special type of biomaterial. They are polyesters, produced by a range of microbes, cultured under different nutrient and environmental conditions [5]. These polymers, which are usually lipid in nature, are accumulated as storage materials (in the form of mobile, amorphous, liquid granules), allowing microbial survival under stress conditions [6,7]. The number and size of the granules, the monomer composition, macromolecular structure and physico-chemical properties vary, depending on the producer organism [8–11]. They can be observed intracellularly as light-refracting granules or as electronlucent bodies that, in overproducing mutants, cause a striking alteration of the bacterial shape (Figure 1).

Atomic force microscopy and confocal Raman spectroscopy are techniques currently used for poly(3-hydroxyalkanoate) (PHA)-granule analysis. Bioplastics can be isolated by centrifugation (cell-free extracts) or by solvent extraction (dried intact bacteria) with chloroform, trifluoroethanol, dichloroethane, propylene carbonate, methylene chloride or dichloroacetic acid [12–15]. Their molecular weights (ranging from 50 000 to 1 000 000 Da) have been established by light scattering, gel permeation chromatography, sedimentation analysis and intrinsic viscosity measurements [5,13,16,17]; and their monomer compositions have been determined by gas chromatography (GC), mass spectroscopy (MS) and nuclear magnetic resonance (NMR) analyses [18]. Other physical properties, such as crystal structure, polydispersity, melting temperature, enthalpy of fusion, glass transition temperature and mechanical properties were established using different procedures [13,16–19].

Most known bioplastics contain, as monomers, different β-oxidation intermediates [(*R*)-3-hydroxyacyl–CoAs], which are enzymatically polymerised by the condensation of the carboxy function, present in a monomeric–CoA thioester with the 3-hydroxy group (or the thiol group) of the next one [1,3•,5,7]. Other bioplastics, containing unusual monomers (e.g. 4-, 5- or 6-hydroxyalkanoic acids or glutamic acid), are synthesised through different pathways [18–23], suggesting that

Figure 1



Scanning **(a,b)** and transmission **(c,d)** electron microphotographs of *P. putida* U **(a,c)** and its Δ*fadBA* β-oxidation mutant **(b,d)** cultured in a chemically defined solid medium containing 7-phenylheptanoic acid (5 mM) as a source of aromatic PHAs and 4-hydroxyphenylacetic acid (5 mM) as an energy source. Bar = 1 μm.

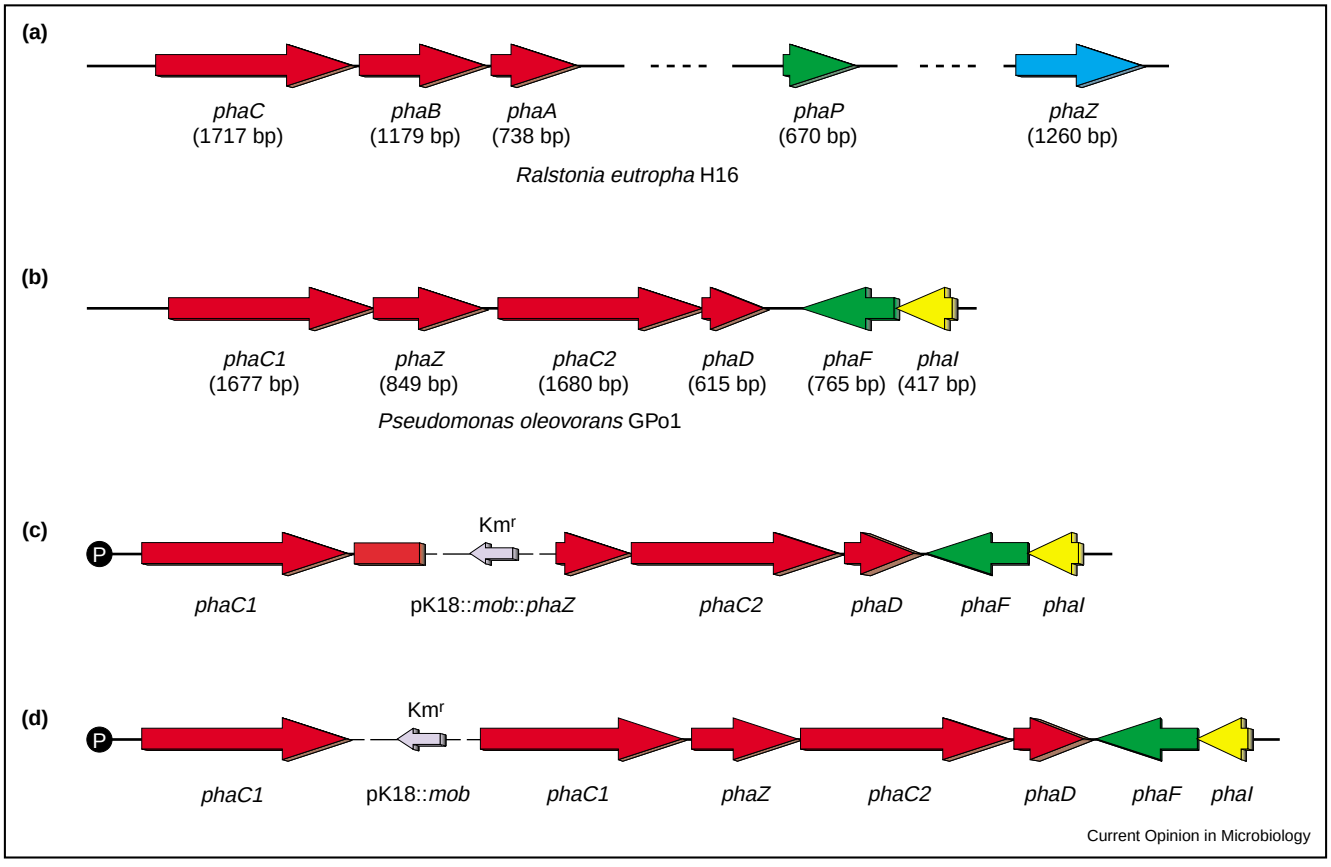
biosynthetic enzymes are widely distributed, and that the production of a particular type of polyester is a strain-specific event.

Currently, the main limitations for the bulk production of bioplastics are its high production and recovery costs [5,8,19,24]. However, genetic and metabolic engineering has allowed their biosynthesis in several recombinant organisms (other bacteria, yeasts or transgenic plants) [25,26,27••,28••], by improving the yields of production and reducing overall costs [1,3•,5,7,8,19,29,30••].

Lemoigne first described a bioplastic — poly(3-hydroxybutyrate) (PHB) in *Bacillus megaterium* [31]. This initial observation was almost forgotten until the mid-1970s when, because of the petroleum crisis, a scientific movement aimed at discovering alternative sources of fossil fuel reserves was undertaken. However, the structure, biosynthetic pathways and applications of many bioplastics have now been established. Microbes belonging to more than 90 genera — including aerobes, anaerobes, photosynthetic bacteria, archaebacteria and lower eukaryotes — are able to accumulate and catabolise these polyesters [3•,5,7,18]. The occurrence of bioplastics has been discussed elsewhere [1,2,5,7,32,33].

The most widely produced microbial bioplastics are PHB, PHA and their derivatives [5,7,19]. However, other polyesters can also be produced by microorganisms. Most of them either require similar biosynthetic enzymes or lack current industrial applications, and hence we shall only describe the genes and enzymes involved in the production of PHBs and PHAs. In this review, we discuss the occurrence, biosynthesis, catabolism and biotechnological applications of poly 3-hydroxyalkanoates.

Figure 2



Organisation of the genes and enzymes involved in the biosynthesis of bioplastics. **(a)** Biosynthesis of PHBs in *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) **(b)** PHAs in *Pseudomonas oleovorans* **(c,d)** PHAs in different mutants of *P. putida* U designed to prove the existence of promoters downstream from *phaC1* (c) *P. putida* U mutant disrupted by the insertion of the integrative plasmid pK18::*mob* into the depolymerase gene (d) *P. putida* U mutant in which the *phaC1* gene has been duplicated and a new cluster *phaC1ZC2DFI*, without the promoter region (P_1) located upstream from *phaC1*, has been generated.

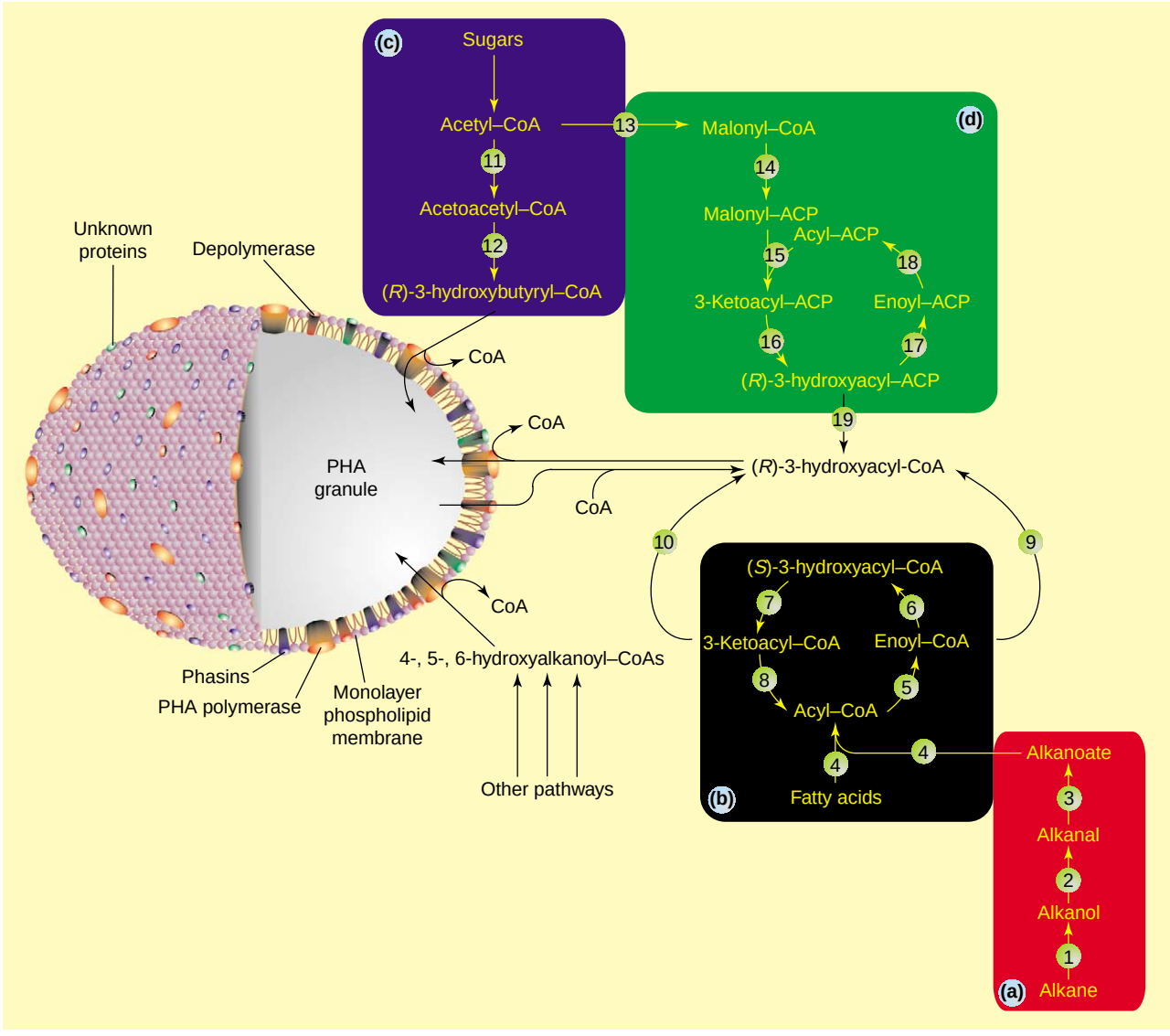
Poly(3-hydroxybutyrate) biosynthesis, catabolism and regulation

The organisation of PHB metabolic genes in *Ralstonia eutropha* is shown in Figure 2. The *phaCBA* cluster encodes three proteins: PhaA (β-ketothiolase), which catalyses the synthesis of acetoacetyl–CoA from acetyl–CoA; PhaB (NADPH-oxidoreductase), which stereospecifically reduces acetoacetyl–CoA to (*R*)-3-hydroxybutyryl–CoA; and PhaC (PHB polymerase), which promotes the incorporation of (*R*)-3-hydroxybutyryl–CoA enantiomers in the growing polymer (Figure 3). PhaC is very active towards monomers containing less than five carbon atoms, although it also synthesises polymers containing small quantities of higher-length monomers (C6–C8) [3•,7,34,35].

The regulation of the PHB pathway seems to be complex. An excess of acetyl–CoA reduces the synthesis of PHBs, whereas all the metabolic or environmental conditions that cause a reduction in the pool of acetyl–CoA start, or restore, PHB synthesis [3•,36,37]. Furthermore, *B. megaterium* PhaC is synthesised as an inactive protein that requires a different polypeptide (PhaR) to be converted into a functional enzyme [38], suggesting that PHB regulation involves different environmental, metabolic and genetic signals [5].

Two additional proteins, PhaZ and PhaP (*phaZ* and *phaP* gene products, Figure 2), also participate either in the catabolism (PhaZ) or in the stabilisation (PhaP) of the PHB granule. PhaZ is a depolymerase (structurally related to esterases) that catalyses the release of (*R*)-3-hydroxybutyrate from the polymer (or from oligomers longer than dimers) (Figure 3) [39,40•]. In the absence of PHB, PhaZ is produced as an inactive protein that requires PHB and an activator (which could be replaced by trypsin) to be transformed into an active enzyme [41]. These observations suggest that either PhaZ is synthesised as a proenzyme, or the attack of PhaZ to the granule surface requires the participation of a proteolytic enzyme

Figure 3



Structural organisation of a PHA granule and metabolic interconnections between the different pathways involved in the biosynthesis and catabolism of PHBs and PHAs. **(a)** Alkane oxidation pathway. (1) Alkane 1-monooxygenase, (2) alcohol dehydrogenase, (3) aldehyde dehydrogenase. **(b)** Fatty-acid β-oxidation. (4) acyl–CoA ligase, (5) acyl–CoA dehydrogenase, (6) enoyl–CoA hydratase, (7) 3-hydroxyacyl–CoA dehydrogenase, (8) 3-ketothiolase, (9) (*R*)-enoyl–CoA hydratase, (10) 3-ketoacyl–CoA reductase. **(c)** Biosynthesis from carbohydrates. (11) β-ketothiolase, (12) NADPH-dependent acetoacetyl–CoA reductase. **(d)** *De novo* fatty acid synthesis. (13) acetyl–CoA carboxylase, (14) ACP-malonyltransferase (15) 3-ketoacyl-ACP synthase, (16) 3-ketoacyl-ACP reductase, (17) 3-hydroxyacyl-ACP reductase, (18) enoyl-ACP reductase, (19) 3-hydroxyacyl-ACP–CoA transacylase.

[3•]. Recent studies have shown that the degradation of PHBs is a complex mechanism that requires several depolymerases (PhaZ1, PhaZ2 and PhaZ3) together with other as yet uncharacterised enzymes [39].

PhaP (phasin) is a low-molecular-weight protein (accumulated to high levels during PHB synthesis) that enhances PHB production by binding to the granules (it regulates the size, number and surface to volume ratio of PHB inclusions) [1,3•,5,7]. Recently, it has been reported that the synthesis and accumulation of PhaP is a PHB-dependent mechanism involving the participation of PhaR (an autoregulated repressor) [42••,43]. However, regulation of the size and number of PHB inclusions is not only modulated by PhaP but also by the quantity of PhaC present in the cells. Thus, in recombinant bacteria,

an excess of polymerase leads to the formation of a large number of PHB inclusions containing low-molecular-weight polymers, whereas low levels of PhaC involves the formation of few granules with higher-molecular-weight polymers [3•,44].

The extracellular degradation of PHB is performed by certain microbial depolymerases (structurally related to hydrolases), as well as by many environmental factors [7,45].

Polyhydroxyalkanoate biosynthesis, catabolism and regulation

PHAs are polyesters containing monomers of medium-chain length (mclPHAs, C5–C14) or long-chain length (lclPHAs, >C14) (see Table 1). Although PHAs are structurally related to PHBs (short-chain length, sclPHAs) [46,47], the microbes that synthesise PHBs usually fail to make PHAs. However, recombinant organisms containing mixed catabolic pathways are able to synthesise either polymers (or co-polymers) containing scl, mcl monomers, or both [3•,7,33–35,48].

The organisation of the mclPHA biosynthetic genes in *Pseudomonas oleovorans* and in *P. putida* is shown in Figure 2. The *phaC1ZC2D* operon encodes two polymerases (PhaC1 and PhaC2), a depolymerase (PhaZ) and the PhaD protein [49,50]. The two polymerases, which are members of the α/β hydrolase subfamily, catalyse the condensation into PHAs of several (*R*)-3-hydroxy-acyl–CoA derivatives (saturated, unsaturated, linear, cyclic, branched or substituted with different functions such as halogen atoms, hydroxy, cyano, carboxy, or phenyl groups) whose side chains range between C5 and C14 atoms [7,12,47,50–54,55•]. Both enzymes are quite similar in their amino acid sequence (about 50%) and substrate specificity (3-OH-acyl–CoA derivatives and 4-, 5- or 6-OH-acyl-CoAs) [5,56], although when expressed in foreign hosts, they are also able to polymerise other monomers [5,7,33–35,50]. Identification of the amino acid residues required for catalysis [57] has allowed modification of the catalytic rates in several PHA synthases [58].

Most mclPHA intermediates are obtained through fatty-acid β-oxidation [47], although other monomers (synthesised from different carbon sources) can also be obtained via different pathways (Figure 3). The use of one or the other seems to be a strain-specific trait [12].

A different type of polymerase (type III) exists in *Chromatium vinosum*, *Thiocystis violacea*, *Thiocapsa pfennigii* and *Synechocystis* sp. PCC 6803 [7]. This is formed by PhaC and PhaE, which mainly synthesise sclPHAs but which also polymerise scl and mcl monomers [7,50]. The existence of two polymerases in the same microorganism (probably as a consequence of gene duplication) represents an interesting evolutionary event that could have contributed to the biochemical transition from PHBs (which only require a single polymerase) to PHAs (where two enzymes are involved). Further studies are needed to confirm this hypothesis.

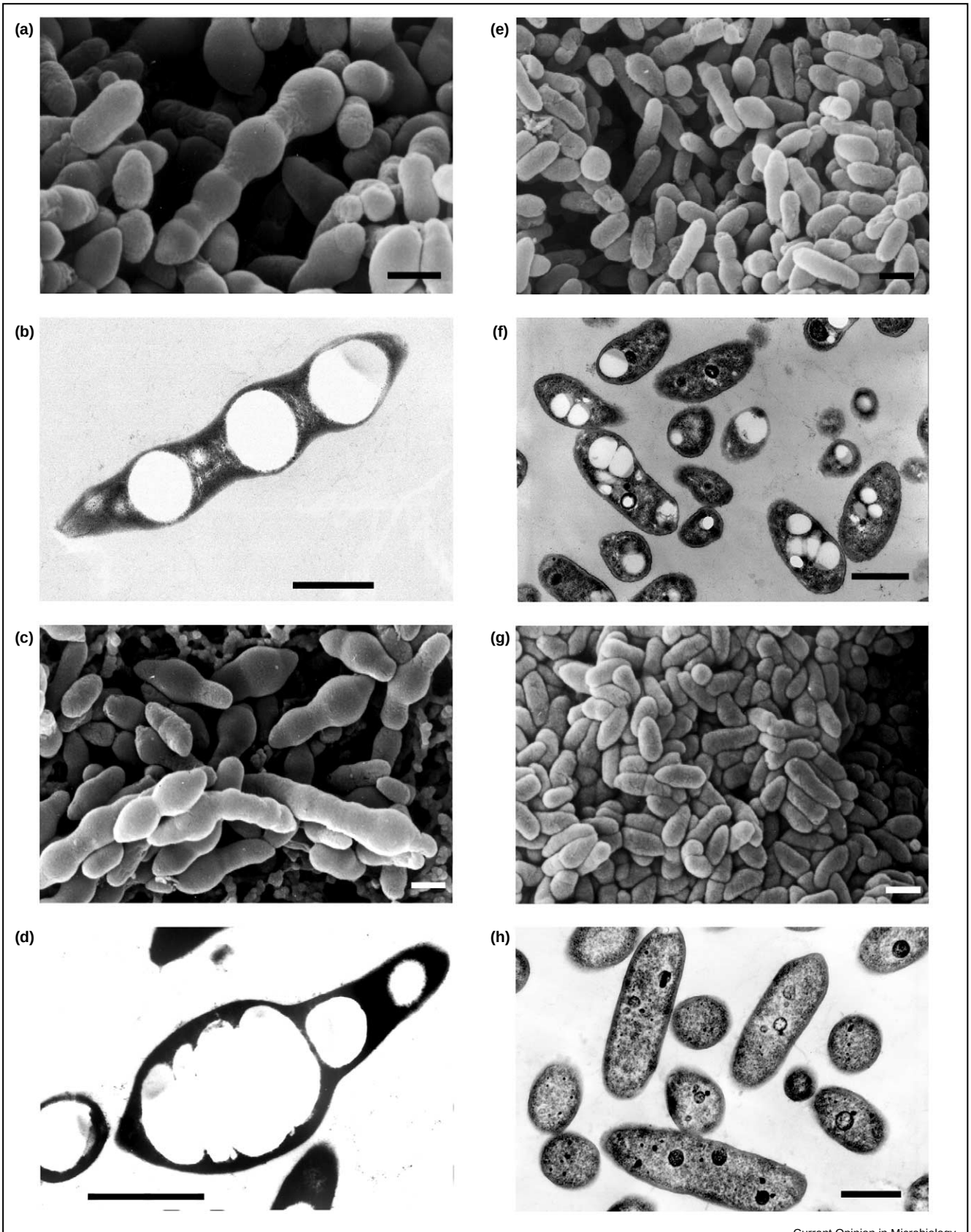
phaC1 and *phaC2* are separated by a third gene that encodes the PhaZ product (Figure 2). This protein, containing a conventional lipase box [3•,4,5,7,50], shows a certain homology with depolymerases (enzymes involved

Table 1

Classification of microbial bioplastics according to different criteria.

Biosynthetic origin	Natural bioplastics: those produced by microorganisms from general metabolites (i.e. PHBs and aliphatic PHAs). Semisynthetic bioplastics: those that require the addition to the culture broth of some precursors that cannot be synthesised by the microbe (i.e. PHAs containing aromatic monomers) Synthetic bioplastics: those polyesters that resemble the natural ones but that can only be obtained by chemical synthesis (i.e. synthetic thermoplastic polymers)
Chemical nature of the monomers	Bioplastic containing aliphatic fatty acid derivatives: saturated or unsaturated (with double or triple bonds) monomers; linear or branched monomers; substituted or not (with functional groups in the monomers). Bioplastics containing aromatic fatty acid derivatives Bioplastics containing both aliphatic and aromatic fatty acid derivatives Bioplastics containing other different compounds (e.g. poly-γ-glutamic acid, poly-ε-L-lysine, poly-β-L-malic acid, polyglycolic acid, cianophicin)
Monomer size	Bioplastics containing a short-chain length (sclPHB and derivatives sclPHAs; C3–C5 monomers) Bioplastics containing a medium-chain length (mclPHAs; C6–C14) Bioplastics containing a long-chain length (lclPHAs; >C14)
Number of monomers in the polyesters	Homopolymeric bioplastic: a single monomer is present in the bioplastic Heteropolymeric bioplastic (copolymer): more than one monomer is present in the bioplastic
Type of polyesters accumulated by the microbe	Unique (a single bioplastic) More than one (mixed bioplastics)

Figure 4



in the mobilisation of sclPHAs) and with many hydrolytic enzymes, suggesting that it participates in the release of hydroxyacyl–CoA derivatives from PHAs. The topological localisation of PhaZ (granule surface) [3•,7] and the inability of certain bacteria to mobilise mclPHAs when *phaZ* is mutated strongly supports its physiological function [5,12].

Expression of the *phaC1ZC2D* cluster in *P. putida* U must be under the control of promoter sequences located upstream of *phaC*; otherwise, it would not be possible to account for the drastic reduction in PHA synthesis that occurs when *phaZ* is disrupted, or when a plasmid is introduced between the duplicated copies of *phaC1* (Figure 2) [1,3•,12].

An additional cluster (*phaFI*), also involved in the biosynthesis of PHAs, is located downstream from the *phaC1ZC2D* operon. These genes encode phasins PhaF and PhaI. PhaF, a histone-H1-like protein, plays a dual function: it is involved in the stabilisation of the granule, and it acts as a regulator [59]. PhaF is a granule-associated protein that represses *phaC1ZC2D* and *phaI* and contributes to the stabilisation of PHA granules; whereas PhaI, another granule-associated protein, only participates in the formation and stabilisation of PHA inclusions [59]. In the absence of PHAs precursor, *phaC1ZC2D* and *phaI* are not expressed, whereas when mcl monomers are synthesised, PhaF is removed from the DNA, initiating (or restoring) PHAs production. Under these new conditions, PhaF and PhaI interact with the hydrophobic nascent polymeric chains, contributing (in an isolated fashion or as a complex) to granule formation [59].

The physiological role of PhaD remains obscure. It is not a granule-associated protein, although it seems to be required for PHA formation [60]. Very recently, we have observed that the deletion of *phaDFI* in the overproducing mutant *P. putida* U Δ*fadBA* causes a considerable reduction (>70%) in the synthesis of aliphatic PHAs, whereas poly(3-hydroxyphenylalkanoates) are not produced (Figure 4). Furthermore, the restoration of PHA synthesis in this double-deleted mutant (Δ*fadBA*Δ*phaDFI*) requires the expression of *phaF*, whereas in its absence, even when *phaD* and *phaI* are expressed, this effect was not reversed (Sandoval *et al.* unpublished data).

Macromolecular architecture of the inclusions

PHAs are accumulated intracellularly (as amorphous mobile polymers) in granules of different sizes (Figure 1). They are surrounded by a phospholipid monolayer (PM) containing phasins PhaF and PhaI [3•,7,50,59,61], polymerases, a depolymerase and cytosolic proteins non-specifically attached to the granule (Figure 3) [3•,7]. The function of the PM envelope has not yet been well established, although it is believed that it is needed to avoid the contact of PHAs with water (preventing the transition of the polyester from the amorphous liquid state to a more stable crystalline form) [7], and that it acts as a protective barrier (avoiding cellular damage caused by the interaction of PHAs with internal structures or with cytosolic proteins) [12,62]. If the PM is indeed required to protect cells from the very beginning of granule formation, it can be assumed that this envelope must be extended around the granule as long as it is increasing in size. Therefore, enzymes specifically involved in the synthesis of the PM, associated with the polyester, must exist. Jurasek *et al.* [63•] have published an interesting discussion on granule formation.

Industrial production

There are three important limitations in the bulk production of bioplastics: first, the special growth conditions required for the synthesis of these compounds (usually unbalanced nutrient conditions that cause slow growth); second, the difficulty involved in synthesising them from inexpensive precursors; and third, the high cost of their recovery [8,24]. However, current knowledge about their biosynthetic pathways and regulation has allowed the construction of recombinant organisms (other microbes, yeasts and plants) able to synthesise bioplastics from inexpensive carbon sources (e.g. molasses, sucrose, lactose, glycerol, oils and methane) [1,2,5,27••,64]. Currently, traditional fermentations carried out with recombinant bacteria and transgenic plants cannot compete with the conventional industrial production of synthetic plastics [1].

A different production strategy is the enzymatic synthesis of bioplastics [1,19]. Although their production in the laboratory is economically inadvisable to date, the characterisation of their biosynthetic enzymes, as well as knowledge about the energetic requirements for such processes, could facilitate their scaling-up and hence the production of new or modified bioplastics in bioreactors [64].

Biotechnological applications

Many different applications have been described for bioplastics since the first industrial production of Biopol® by ICI Ltd in 1982. Initially, they were used for the fabrication of bottles, fibres, latex and several products of agricultural, commercial or packaging interest [1,2]. Currently, these polyesters have been employed for medical

(Figure 4 Legend) Scanning **(a,c,e,g)** and transmission **(b,d,f,h)** electron microphotographs of the *P. putida* U Δ*fadBA* β-oxidation mutant **(a–d)** and its Δ*fadBA*Δ*phaDFI* double-deleted mutant **(e–h)** cultured in a chemically defined solid medium containing either octanoic acid (5 mM) **(a,b,e,f)** or 7-phenylheptanoic acid (5 mM) and 4-hydroxyphenylacetic acid (5 mM) **(c,d,g,h)**. Bar = 1 μm.

applications such as sutures, implants, urological stents, neural- and cardiovascular-tissue engineering, fracture fixation, treatment of narcolepsy and alcohol addiction, drug-delivery vehicles, cell microencapsulation, support of hypophyseal cells, or as precursors of molecules with anti-rheumatic, analgesic, radiopotentiator, chemopreventive, antihelmintic or anti-tumuoral properties (those containing aromatic monomers or those linked to nucleosides) [1,2,7,19,54,55•,64–67]. The specific properties needed for the applications of bioplastics have been widely discussed [2].

Conclusions

To date, more than 160 different polyesters with plastic properties have been described and this number is growing exponentially by means of genetic and metabolic engineering techniques. The collection of novel polyesters using recombinant microbes suggests that the biosynthetic limitations observed in the original strain are not imposed by a strict substrate specificity of the anabolic enzymes but, instead, are due to other physiological reasons. Thus, it could be expected that many other bioplastics with different structures, properties and applications could be obtained if the appropriate organism were selected and genetically manipulated.

In conclusion, because of their special characteristics and broad biotechnological applications, bioplastics are compounds with an extremely promising future.

Acknowledgements

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Although many other excellent reports have been published about this topic, here we only include those ones that, in our opinion, would be required to provide current information for general readers. We apologise that space limitations do not permit the citation of many other interesting contributions.

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