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# Engineering Native and Synthetic Pathways in *Pseudomonas putida* for the Production of Tailored Polyhydroxyalkanoates

Mariela P. Mezzina, María Tsampika Manoli, M. Auxiliadora Prieto, and Pablo I. Nikel\*

This article is dedicated to the memory of our colleague, Dr. Shane Kenny, who made significant contributions toward developing bioprocesses for sustainable PHA production. The scientific community is deeply saddened by his untimely demise in 2020.

Growing environmental concern sparks renewed interest in the sustainable production of (bio)materials that can replace oil-derived goods. Polyhydroxyalkanoates (PHAs) are isotactic polymers that play a critical role in the central metabolism of producer bacteria, as they act as dynamic reservoirs of carbon and reducing equivalents. PHAs continue to attract industrial attention as a starting point toward renewable, biodegradable, biocompatible, and versatile thermoplastic and elastomeric materials. *Pseudomonas* species have been known for long as efficient biopolymer producers, especially for medium-chain-length PHAs. The surge of synthetic biology and metabolic engineering approaches in recent years offers the possibility of exploiting the untapped potential of *Pseudomonas* cell factories for the production of tailored PHAs. In this article, an overview of the metabolic and regulatory circuits that rule PHA accumulation in *Pseudomonas putida* is provided, and approaches leading to the biosynthesis of novel polymers (e.g., PHAs including nonbiological chemical elements in their structures) are discussed. The potential of novel PHAs to disrupt existing and future market segments is closer to realization than ever before. The review is concluded by pinpointing challenges that currently hinder the wide adoption of bio-based PHAs, and strategies toward programmable polymer biosynthesis from alternative substrates in engineered *P. putida* strains are proposed.

## 1. Introduction

Growing global environmental concerns urgently call for smart alternatives to the use of oil-derived commodities to reduce our dependency on limited fossil resources—while limiting pollution and CO<sub>2</sub> emissions. Among the countless oil-derived commodities we rely on, plastics are in the spotlight as highly recalcitrant polymers produced at a high scale and extensively used for a wide range of consumer and industrial applications. In 2017, global plastic production reached ≈350 million tons. As reported in recent market studies by Plastics Europe (PEMRG, <https://www.plasticseurope.org/en>) and the Conversio Market and Strategy GmbH (<https://www.conversio-gmbh.com/en>), China is the biggest producer of plastics worldwide (accounting for 50.1%), followed by Europe (18.5%) and North America (17.7%). The massive production of oil-derived plastics and inappropriate waste management strategies during the last decades inevitably led to significant plastic pollution of lands

and oceans. Plastic wastes reach ≈5–13 million tons per year, mostly accumulating in marine environments and causing severe environmental damage.<sup>[1]</sup> Furthermore, plastic production has a direct impact on greenhouse gas emissions<sup>[2]</sup> and thus contributes to global warming and climate change.

Biobased plastics have arisen as an alternative to traditional plastics as they present similar properties and offer additional advantages over petrochemical materials, e.g., reduced carbon footprint and the broader availability of waste management options.<sup>[3]</sup> Biobased plastics are suited for a range of end-of-life alternatives,<sup>[4]</sup> including reuse, mechanical and organic recycling, and energy recovery.<sup>[5]</sup> These materials comprise “drop-in” polymers (chemically identical to their petrochemical counterparts, but derived from biomass and often nonbiodegradable), and compostable and/or biodegradable plastics such as polyhydroxyalkanoates (PHAs), polylactic acid, and starch blends.<sup>[6]</sup> The latter group of polymers is mainly used for short-lived applications (e.g., packaging) as well as coatings and adhesives (European Bioplastics, <https://www.european-bioplastics.org>). In 2018, the European Commission published its strategy

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for plastics in a circular economy—proposing, among other performance targets, that all plastic packaging should be designed to be fully recyclable or reusable to close the loop of plastic losses and to reduce marine litter deposits.<sup>[7]</sup> Two further promising initiatives of the European Commission encompass a 90% collection target for plastic bottles by 2029 and the ban on single-use plastics that has been recently approved by the European Parliament (expected to be in force by 2021). Under these new rules, single-use plastic items such as plates, cutlery, straws, balloon sticks, and cotton buds will be totally banned from the market. Political action notwithstanding, numerous challenges are to be overcome in the fight against plastic pollution—e.g., the quality of recycled plastics is increasing, but the overall uptake of recycled plastics remains low. A glaring consequence of this problem is that incineration of plastic wastes gives rise to ≈400 million tons CO<sub>2</sub>/year globally<sup>[8]</sup> and these figures could be further enlarged by the ongoing COVID crisis.<sup>[9]</sup> In this context, establishing a market of biobased plastics (e.g., PHAs) is seen as a prime solution in terms of bioeconomy.<sup>[10]</sup> Recent economic studies revealed that biobased polymers will create new business opportunities, displaying stable growth in emerging plastic markets.<sup>[11]</sup> In 2019, the global production of biobased plastics amounted to 2.1 million tons—still representing a mere 1% of the total plastic produced annually. As the demand is rising (and since regulatory frameworks look permissive), the bioplastic production capacity is set to increase to >2.4 million tons by 2024. Furthermore, the development of novel and improved biopolymers and new applications drives the growth and diversification of the market. On the one hand, innovative biopolymers (e.g., biobased polypropylene and PHAs) show the highest relative growth rates as of today. PHA production capacities, on the other hand, are estimated to grow by >3 times in the next 5 years. In contrast to other commercially available biopolymers, PHAs are 100% biobased and biodegradable, and feature a wide diversity of physical and mechanical properties that could fulfill the material demands of such an expanding market.

Bacterial cell factories for PHAs production have been extensively reported in the literature over the last three decades. Among them, *Pseudomonas* species constitute an excellent model to study and manipulate PHA biosynthesis from a wide variety of substrates. *Pseudomonas putida*, for instance, is a natural PHA producer considered as a model bacterium for biodegradation studies and a production platform, mainly because of its high tolerance to solvents and oxidative stress conditions.<sup>[12]</sup> Such properties, together with a plethora of synthetic biology (SynBio) tools tailored for targeted genetic manipulations developed in the last years<sup>[13]</sup> and the availability of no less than eight genome-scale metabolic models (GSMMs) for *in silico* studies,<sup>[14]</sup> position *P. putida* as one of the preferred SynBio chassis.<sup>[15]</sup> Such unique features are of interest for several technical applications, e.g., production of complex molecules,<sup>[16]</sup> degradation of contaminants,<sup>[17]</sup> and, importantly, biosynthesis of tailor-made biopolymers.<sup>[18]</sup> Since *P. putida* exhibits a remarkable capability to breakdown aromatic compounds,<sup>[19]</sup> many of them being recalcitrant pollutants of their own, this host offers unique opportunities for the upcycling of contaminants into value-added polymers.

On this background, this review article discusses the role of native, core metabolic pathways in PHA biosynthesis from substrates displaying different chemical nature, and the regulatory factors known to affect polymer accumulation. Next, we summarize key metabolic engineering, SynBio, and systems biology approaches to engineer the endogenous PHA machinery for optimizing polymer biosynthesis and incorporating novel chemical groups and atoms. We focus on the biochemical mechanisms that remain unexplored targets for manipulation and on the interplay of factors that drives biopolymer production (an aspect that eluded a comprehensive understanding of PHA metabolism until recent times). Furthermore, we discuss how programming *Pseudomonas* cell factories to obtain novel PHAs via smart SynBio and metabolic engineering will be key toward obtaining novel biopolymers that bring about new-to-nature physical and mechanical properties—thus impacting processability and material applications.

## 2. General Aspects of Bacterial Metabolism of PHA and Physicochemical Properties of the Materials

PHAs are a family of structurally diverse storage polyesters accumulated by many bacterial species as carbon and energy reserve materials. Since their discovery in the early 1900s, PHAs have been recognized as a green alternative to the currently used plastics derived from petroleum, since some of these biopolymers possess physical and thermochemical properties comparable to traditional, oil-based materials. PHAs are also biocompatible and can be produced sustainably from agroindustrial waste materials or low-cost substrates using bacterial cell factories. Moreover, these materials are widely recognized as biodegradable and compostable—a feature that positions PHAs as a potential solution for alleviating the growing plastic waste global crisis. Decades of research on the topic expanded our understanding of the biochemical pathways, metabolic regulation, and ecological significance of bacterial PHAs.

The biosynthesis of bacterial polyesters is a response to unbalanced growing conditions, characterized by a high supply of carbon and limitation of an inorganic nutrient, e.g., nitrogen, oxygen, or phosphorous. Bacteria accumulate PHAs as intracellular granules.<sup>[20]</sup> The insoluble and highly hydrophobic inclusions where PHAs accumulate are often referred to as carbonosomes, as they are composed of the polymer proper and an organized layer of granule-associated proteins (GAPs) that play structural, biosynthetic, catabolic, and regulatory functions. GAPs encompass PHA synthases, depolymerases, and a group of low-molecular-weight proteins called phasins. Other GAPs can also be found on the surface of the granule, e.g., transcriptional regulators, hydrolases, reductases, or acyl-coenzyme A (CoA) synthetases<sup>[21]</sup>—although the role of some of them is yet to be defined. PHA synthases, however, are essential GAPs, as they are key for polyester biosynthesis. Synthases transform (*R*)-3-hydroxyacyl-CoA [(*R*)-HA-CoA] units into a polyester chain by releasing one CoA molecule per catalytic cycle. The resulting molecule can be further degraded into free (*R*)-HA monomers by PHA depolymerases (yet another type of GAP) to fulfill metabolic demands. Phasins, on the other hand, are amphipathic proteins



located in the interphase between PHA and the cytoplasm, and these structural GAPs play regulatory and functional roles, e.g., modulating the formation, localization, number, and size of the polymer granules and granule segregation during cell division.<sup>[22]</sup> These macromolecular organization traits are largely conserved across species no matter the chemical nature of the PHA accumulated.

From a structural point of view, PHAs are classified into three large families according to the total number of carbon atoms in the (*R*)-HA monomers: i) short-chain-length (scl-PHA), poly(3-hydroxybutyrate) (PHB, a homopolymer composed by C4 monomers) being the most abundant example; ii) medium-chain-length (mcl-PHA), having monomers ranging from C6 to C14; and iii) long-chain-length (lcl-PHA), composed by C15 (or longer), scarcely found in Nature.<sup>[23]</sup> Whereas several bacteria produce scl-PHA, including the model species *Cupriavidus necator* (formerly known as *Ralstonia eutropha*),<sup>[24]</sup> mcl-PHA producers belong mainly to the genus *Pseudomonas*.<sup>[25]</sup> The monomer composition defines most of the physicochemical properties of the material and this property is a key target for engineering efforts, as it will be discussed later. Besides, depending on the bacterial species and the substrates provided, PHAs can be synthesized as homopolymers (i.e., presenting only one type of monomer), random copolymers (i.e., with two or more different monomers) and block copolymers (i.e., composed by at least two homopolymers connected by covalent bonds). Monomers are named according to the number of carbons in their structure, e.g., 3HB, 3-hydroxybutyrate (C4); 3HV, 3-hydroxyvalerate (C5); 3HHx, 3-hydroxyhexanoate (C6); 3HHp, 3-hydroxyheptanoate (C7); 3HO, 3-hydroxyoctanoate (C8); 3HD, 3-hydroxydecanoate (C10); 3HDD, 3-hydroxydodecanoate (C12); and 3HTD, 3-hydroxytetradecanoate (C14).

PHAs are semicrystalline polymers, therefore, the overall thermal properties are generally expressed in terms of the glass transition temperature ( $T_g$ ) of the amorphous phase and the melting temperature ( $T_m$ ) of the crystalline phase. Both thermal parameters are highly dependent on the monomer composition. In the amorphous region, the molecules of the polymer are in a frozen state at low temperatures, allowing for slight vibration of molecules. In this state, commonly referred to as a glassy state, the polymer tends to be brittle and rigid, analogous to a glass. If the polymer is heated up, the molecules start to move, and the polymer becomes flexible—leading to the “rubbery” state since the material acquires properties similar to rubber. Hence,  $T_g$  defines the transition from the glassy state to the rubbery state of the amorphous fraction of the polymer. All polymers present a  $T_g$  value, whereas the  $T_m$  is a characteristic of semicrystalline polymers and its presence is directly related to the crystalline fraction of the material. In these polymers, the  $T_m$  marks the transition from the ordered phase to the disordered phase. Although this is a general characteristic of conventional PHAs, incorporation of some types of modified monomers produced amorphous PHAs with no crystalline region and thus, no  $T_m$ —as it will be shown later in this review. These properties can be easily illustrated by examples of naturally-occurring PHAs. scl-PHAs are stiff and brittle polyesters of high crystallinity with thermal and mechanical properties comparable to those of polypropylene (e.g., PHB has a  $T_m = 173\text{--}180\text{ }^\circ\text{C}$  and a  $T_g = 5\text{--}9\text{ }^\circ\text{C}$ ), whereas mcl-PHAs possess lower  $T_m$  and  $T_g$  values [e.g., P(3HHx-co-3HO) 12–88%

(mol/mol) presents a  $T_m = 61\text{ }^\circ\text{C}$  and a  $T_g = -31\text{ }^\circ\text{C}$  and P(3HHx-co-HO-co-HD-co-HDD-co-HTD) has a  $T_m = 58.1$  to  $66.8\text{ }^\circ\text{C}$  and a  $T_g = -42$  to  $-40\text{ }^\circ\text{C}$ , depending on the abundance of each monomer].

The understanding of the mechanical properties of a polymer is also important to evaluate the elasticity and potential applications of the material, with i) tensile strength ( $\sigma^t$ ), the maximum force or tensile stress required to break the polymer by stretching it, and affected by the molecular weight, cross-linking and crystallinity, ii) percent elongation to break ( $\epsilon^b$ ), which measures ductility and is calculated as the change in the length of the material before the break (tensile strain) measured as a percentage (i.e., the values of thermoplastics are over 100%), and iii) Young's modulus ( $E$ ), a measure of the material stiffness within the linear region of the stress–strain curve and calculated as the  $\sigma^t/\epsilon^b$  ratio. In general, mcl-PHAs are characterized by limited crystallinity and large flexibility, and high  $E$  values.<sup>[26]</sup> mcl-PHAs are considered promising candidates as thermoplastic elastomers for packaging and some medical applications not only because of their mechanical and thermal properties but also due to its biocompatibility, hydrophobicity, low oxygen permeability, water-resistance, and industrial compostability.<sup>[25,27]</sup> Since their discovery and industrial production, these polymers have been applied as coatings and in medical temporary implants, e.g., as scaffolding for tissue engineering and as drug carriers.<sup>[28]</sup> Despite these remarkable properties, conventional mcl-PHAs exhibit relatively low  $\sigma^t$  and  $T_m$  values that difficult their processability and limit their use in key applications.<sup>[25]</sup> The need to diversify and improve the physical and mechanical properties of microbial polyesters to expand their potential applications is self-evident.<sup>[29]</sup> One of the most evident transformations to upgrade the properties of natural PHAs is the introduction of functionally diverse chemical groups into the side chains of the polymers—as the  $T_m$  usually increases in the presence of aromatic or large side groups because the flexibility of the lateral chain is directly affected by the presence of such groups.<sup>[30]</sup> The strategies deployed for this purpose will be discussed later in the review, but before delving into the details on how this could be achieved, the natural richness of PHA polymers is presented in the section below.

### 3. PHA Biosynthesis in *Pseudomonas* Species

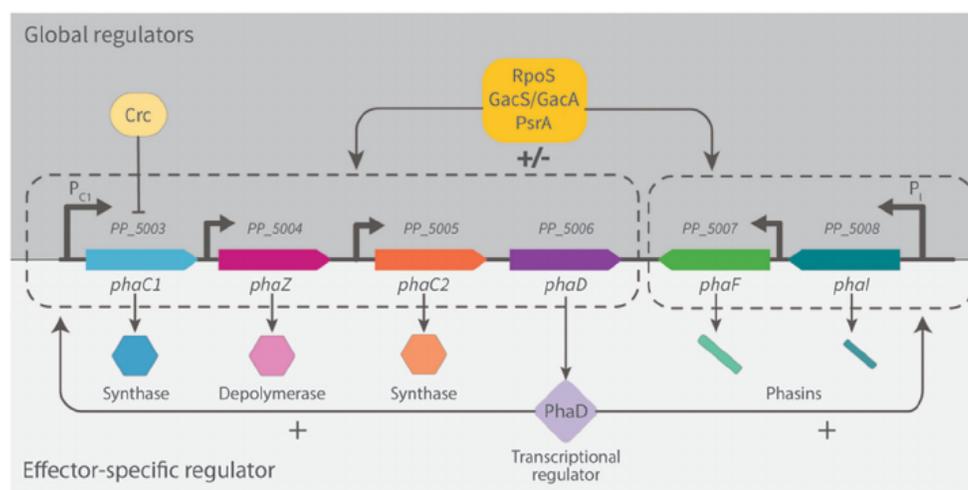
Polymers of the mcl-PHA category isolated from *Pseudomonas* were identified as promising materials early on. Although other bacterial species are also known to accumulate such intracellular inclusions, the scope of this review will be restricted to PHAs produced by *Pseudomonas*, with a focus on *P. putida* KT2440 as a preferred chassis for the manipulation of biopolymer metabolism. The main *Pseudomonas* species reported as PHA producers in the primary literature are summarized in **Table 1**, where it becomes immediately apparent that several natural isolates have been identified as solid candidates for bioplastic production. One such example is the species formerly known as *P. oleovorans*. The intracellular inclusions of *P. oleovorans* (later renamed as *P. putida* GPo1) grown on *n*-octane (i.e., a C8 substrate) as the only carbon source were identified as the first-case occurrence of mcl-PHA. These inclusions are formed by a copolyester consisting of 89% (mol/mol) 3HO and 11% (mol/mol) 3HHx.<sup>[31]</sup> Since then, many other reports showed that the biosynthesis and accumulation



**Table 1.** *P. putida* strains used for the production of PHAs.

<i>P. putida</i> strain <sup>a)</sup>	Description	Ref.
KT2440	Wild-type strain, a derivative of strain mt-2 (originally isolated in Japan as a strain able to degrade <i>meta</i> -toluate <sup>[33]</sup> ) cured of the TOL plasmid pWVO	[34]
EM42	Genome-reduced version of KT2440	[35]
KT2442	Rifampicin-resistant variant of KT2440	[36]
GpP104	PHA-negative mutant of KT2442	[37b]
Gpo1	Isolated from soil; formerly known as <i>P. oleovorans</i> GPo1 (TF4-1L = ATCC 29347)	[38]
27N01	Isolated from soil; identified by the National Collections of Industrial and Marine Bacteria (NCIMB, Scotland)	[39]
U	Isolated from mud in a creek in Urbana, Illinois, USA (British NCIB 10015)	[40]
A514	Lignin-utilizing and plant-associated isolate	[41]
GO16	Isolated from a PET recycling plant	[42]
S12	Styrene-degrading strain isolated from soil	[43]
CA-1	Isolated from a styrene-degradation bioreactor	[44a]
NBUS12	Styrene-degrading strain isolated from a bioreactor seeded with domestic activated sludge from a water reclamation plant	[44b]
CA-3	Isolated from a styrene bioreactor (NCIMB 41162)	[45]
BM01	Isolated from soil in a landfill site	[46]
KCTC 2407	Isolated from river mud in Hull, U.K. (NCIMB 9865)	[47]
KCTC 1639	Identified while screening for mcl-PHA producers from soil samples	[48]
LS46	Isolated from sewage sludge samples from a wastewater treatment plant in Winnipeg, Manitoba, Canada	[49]

<sup>a)</sup> Some of the strains listed have been originally characterized as different species of *Pseudomonas*.



**Figure 1.** Organization of the *pha* gene cluster in *P. putida* KT2440 and transcriptional regulation of individual units in the locus. The regulation exerted on components of the PHA biosynthesis machinery at the transcriptional level is divided into global effects (upper part) and effector-specific effects (lower part). Elements in the diagram are not drawn to scale, and abbreviations are as follows: Crc, catabolite repression control protein; RpoS, stationary phase  $\sigma$  factor; PsrA, a transcriptional regulator of some components of  $\beta$ -oxidation; and GacS/GacA, a two-component global antibiotic and cyanide control system involved in interactions with small regulatory RNAs.

of mcl-PHAs are a general property of *Pseudomonas* species belonging to the rRNA homology group I, which includes both fluorescent and nonfluorescent specimens.<sup>[32]</sup> This property is so conserved across species that it is used as a phylogenetic marker.

The ubiquitous soil bacterium *P. putida* KT2440 produces mcl-PHA copolymers with monomers ranging from C6 to C14 depending on the substrate used. The organization of the *pha* gene cluster in this model organism, which is well conserved among mcl-PHA producer strains, comprises two main operons (Figure 1). The first one encodes two mcl-PHA synthases

or polymerases, PhaC1 (PP\_5003) and PhaC2 (PP\_5005), a depolymerase (PhaZ, PP\_5004), and a transcriptional activator (PhaD, PP\_5006). The second operon, located adjacent to the first one but in the opposite direction, encodes the phasins PhaF (PP\_5007) and PhaI (PP\_5008)—two major GAPs that play important structural and regulatory roles in strain KT2440.<sup>[50]</sup> Besides the presence of *pha* genes that encode both regulatory and structural proteins, the biosynthesis of PHAs in *Pseudomonas* involves a multilayered array of regulatory and biochemical processes. The accumulation of biopolymers is a complex and



global metabolic process that blends the activity of central metabolic pathways (e.g.,  $\beta$ -oxidation and de novo fatty acid synthesis (FAS), which convert intermediates from the catabolism of different substrates into PHA monomers) and a peripheral PHA biosynthetic pathway (further processing monomers and mediating their polymerization). The PHA synthesis route is encoded by the *pha* gene cluster, the products of which direct carbon fluxes toward either PHA accumulation or polymer hydrolysis as an active response to the carbon and energy supply and demand.<sup>[51]</sup> The metabolic and regulatory architecture of the PHA biosynthesis/depolymerization cycle has been described to a significant degree of detail in *P. putida*, where it plays a central role in modulating global carbon metabolism.<sup>[52]</sup> While specific aspects of transcriptional regulation will be discussed later in the article, we start by revisiting the role of  $\beta$ -oxidation as a key source of metabolic intermediates for PHA production in *P. putida* KT2440.

#### 4. $\beta$ -Oxidation Orchestrates PHA Biosynthesis from Structurally Related Substrates

Early works describing PHA synthesis in *Pseudomonas* showed that the composition of the polymers mirrored the structure of the alkanolic acid used as a carbon source. When the substrate consisted of C6 to C12 units, the (*R*)-HA monomers in the PHA polymer either presented the same length or were shortened by exactly C2, C4, or C6 units. Furthermore, when substrates with an even number of carbon atoms in the aliphatic chain (C-even substrates) were used as the carbon source, only C-even monomers were found in the resulting PHA. By contrast, substrates with an odd number of carbon atoms in the aliphatic chain (C-odd substrates) gave rise to PHAs composed of C-odd monomers only (Figure 2). Mixtures of two different PHA-related substrates as carbon sources resulted in the formation of polymers where the monomer composition reflected the ratio of the two carbon sources. The composition of these PHAs and their direct relationship with the structure of the growth substrate suggested that  $\beta$ -oxidation, an essential central pathway of carbon metabolism, played a key role in polymer production from PHA-related substrates.<sup>[20a,32a,53]</sup> The archetypal  $\beta$ -oxidation pathway of *Pseudomonas* species, encoded in the *fad* (for fatty acid degradation) genes, drives the degradation of fatty acids by removing C2 units as acetyl-CoA—explaining the shortage in C2 (and multiples thereof) moieties of PHA monomers introduced in the polymer.

When grown on fatty acids, *P. putida* KT2440 transport these substrates inside the cell utilizing two fatty acid transporters that present different specificities according to the chain length. According to the experimental evidence obtained so far, the first one is a putative long-chain fatty acid transporter encoded by *fadL* (PP\_1689), and the second one is a putative short-chain fatty acid transporter encoded by *PP\_3124*.<sup>[54]</sup> The transport of fatty acids through the cytoplasmic membrane is coupled to their activation to acyl-CoA thioesters. This reaction is catalyzed by FadD, a cytosolic, membrane-associated protein that acts as an ATP-, CoA-, and Mg<sup>2+</sup>-dependent fatty acid-CoA ligase with a broad substrate specificity (Figure 2).

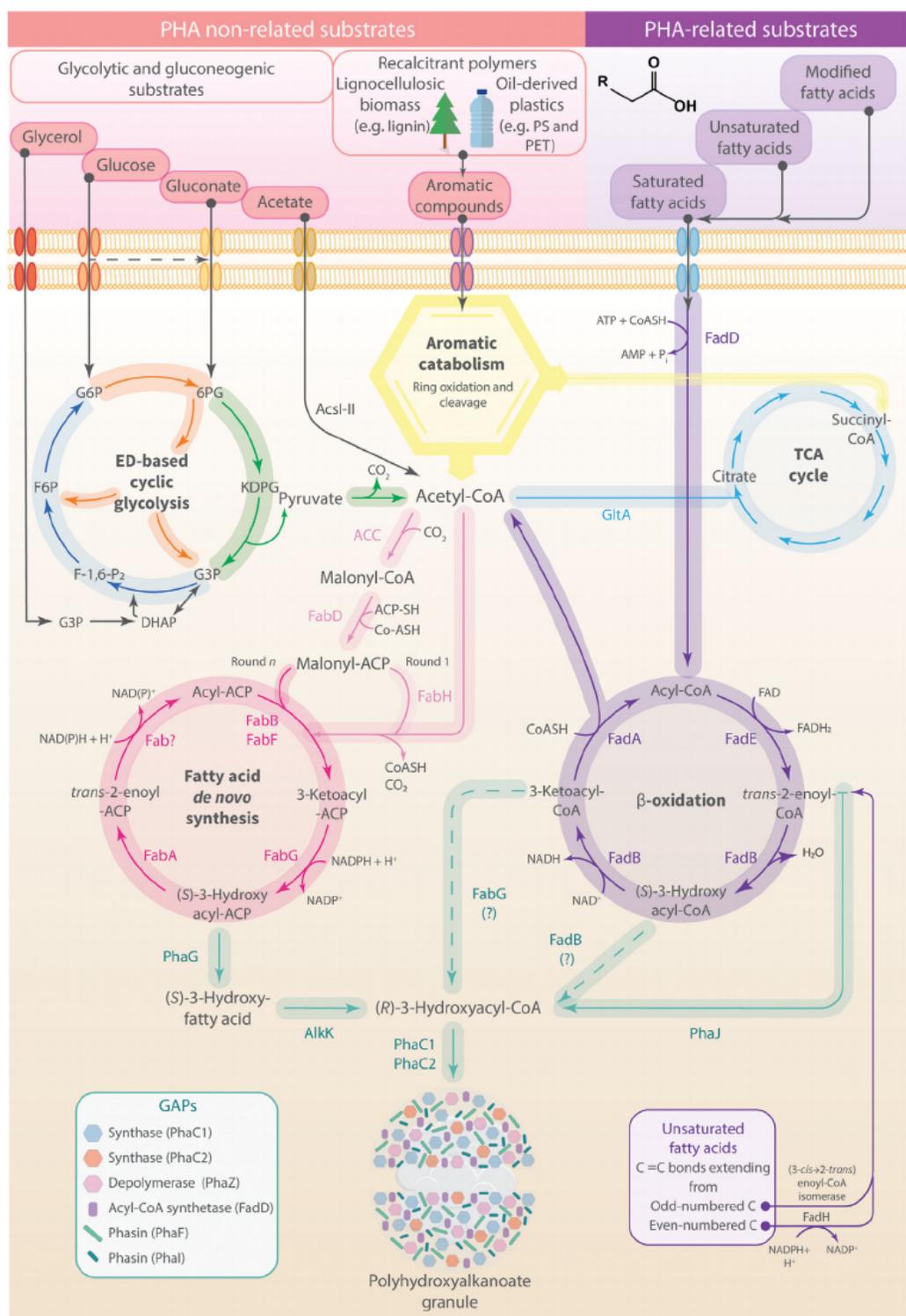
Studies performed in different *P. putida* strains identified two FadD orthologues in *P. putida* U (termed FadD1 and FadD2)<sup>[55]</sup>

and in *P. putida* GPo1 (termed Acs1 and Acs2).<sup>[21b]</sup> A FadD1 deletion mutant of strain U was shown to have a long lag phase when grown on fatty acids as the sole carbon source, while FadD2 seemed to be a cryptic gene, the transcription of which induced when FadD1 was inactivated.<sup>[54,55]</sup> Furthermore, Acs1 and Acs2 shared 94% and 92% identity to FadD1 and FadD2 of *P. putida* U, respectively. Acs1 has been revealed to be associated with PHA granules by fluorescence microscopy experiments, and its role appears to be the activation of (*R*)-HAs released by PHA depolymerase during polymer breakdown. Acs2, by contrast, is located in the cellular membrane.<sup>[21b]</sup> Also, FadD1 from *P. putida* CA-3, showed higher activity on longer chain alkanolic and phenylalkanoic acids than on shorter chain substrates.<sup>[56]</sup> Six putative FadD enzymes were identified in *P. putida* KT2440—among them, FadD1 (PP\_4549) and FadD2 (PP\_4550), which have 94% and 91% identity to FadD1 and FadD2 of *P. putida* U, respectively; and FadDx (PP\_2213), with no evident orthologue(s) in other species. Global fitness analysis of transposon libraries generated by random barcode transposon sequencing (RB-TnSeq) helped to shed light on the role of predicted  $\beta$ -oxidation genes variants when *P. putida* KT2440 is grown with different chain-length fatty acids. This work suggested that FadD1 catalyzes the initial CoA-ligation of medium and long-chain (i.e., C7 to C18) fatty acids (probably acting on C6 as well). Very recently, FadD1 (PP\_4549) has been identified as a prominent GAP in the granule proteome of *P. putida* KT2440—suggesting that biochemical reactions involved in the entire PHA cycle (i.e., polymer synthesis, polymer degradation, and monomer activation) are carried out in the proximity of the granule surface, thus facilitating a continuous and dynamic turnover of PHA.<sup>[57]</sup>

Disruption of FadD2 had no severe effects on bacterial growth, although it did result in moderate fitness defects when cells were grown on C8–C10 fatty acids. These analyses also revealed that two of the predicted CoA-ligases display specificity for short-chain length fatty acids, as the CoA-ligases encoded by *PP\_0763* and *PP\_4559* are required for growth on hexanoate (i.e., a C6 substrate), whereas only *PP\_0763* is required for growth on valerate (i.e., pentanoate, C5).<sup>[58]</sup>

Once produced, an acyl-CoA of *n* carbon atoms enters the first round of  $\beta$ -oxidation and is oxidized to enoyl-CoA with the concomitant reduction of FAD to FADH<sub>2</sub>. This step is catalyzed by an assortment of acyl-CoA dehydrogenases (ACADs) and this conversion constitutes the rate-limiting step of  $\beta$ -oxidation, as these enzymes present the lowest activity described among the components of the pathway (Figure 2). ACADs belong to a large family of flavoproteins and differ in their substrate specificity, with different groups of these enzymes preferring short, medium, long, or even very long acyl-CoA carbon chains.<sup>[59]</sup> According to the latest predictions, 7 ORFs encode putative ACADs in the genome of *P. putida* KT2440.<sup>[58]</sup> Experimental evidence based on the physiological effects of deleting genes encoding putative ACADs indicates that many of these enzymes are either redundant or overlap in terms of function when strain KT2440 is grown on aliphatic fatty acids.<sup>[60]</sup> For instance, a FadE homolog, encoded by *PP\_1893*, displays the highest degree of identity with the well-characterized FadE of *Escherichia coli* and was proposed to actively participate in fatty acid degradation. The deletion of this gene in *P. putida* KT2440 led to decreased PHA synthesis but did not affect the use of fatty acids of different chain lengths





**Figure 2.** Biochemical pathways involved in PHA synthesis in *P. putida* KT2440. The metabolic network for PHA biosynthesis from related and nonrelated substrates is represented in six main metabolic blocks, identified with different colors: (i) ED-based cyclic glycolysis, comprising activities of the EMP pathway (blue), the pentose phosphate pathway (orange), and the ED pathway (green); (ii) catabolic routes for a breakdown of aromatic compounds (yellow); (iii) tricarboxylic acid cycle (TCA cycle, light blue); (iv) de novo fatty acid synthesis (pink, note that biosynthesis of malonyl-ACP from acetyl-CoA and condensation of acetyl-CoA and malonyl-CoA in the first round of the cycle is shown in light-pink); (v)  $\beta$ -oxidation (purple) and (vi) PHA biosynthesis (teal). Key abbreviations are as follows: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; 6PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; G3P, glyceraldehyde-3-phosphate; F-1,6-P<sub>2</sub>, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; CoA, coenzyme A; ACP, acyl carrier protein; GAPs, granule-associated proteins; GltA, citrate synthase; AcsI-II, acetyl-CoA synthase; PET, polyethylene terephthalate; and PS, polystyrene.



as a carbon source. *PP\_2437* encodes an acyl-CoA dehydrogenase that contains a FadE2 domain, demonstrated to play a major role in the metabolism of medium- to long-chain fatty acids because of its high specificity toward these substrates.<sup>[60]</sup> Besides, *PP\_2216*, encoding Acd, has been shown to display specificity for short-chain aliphatic acyl-CoAs<sup>[61]</sup> and the product of *PP\_0368* has been identified as an inducible phenylacetyl-CoA dehydrogenase,<sup>[62]</sup> the role of which became relevant when aromatic compounds are used as substrates. Recent data from global fitness analyses revealed that the *fadE* homolog *PP\_0368* encodes the primary ACAD for fatty acids with chain lengths of C10 and longer, while the nearby *fadE* homolog *PP\_0370* appears to be preferred when C6–C8 fatty acids are fed. Also, a relatively even fitness defect brought about by the deletion of these two *fadE* homologs indicates that they may have equal activity on nonanoate (i.e., C9). *PP\_0370* seems to be the acyl-CoA dehydrogenase largely responsible for hexanoate catabolism, with *PP\_3554* mutants also showing minor fitness defects. Lastly, *PP\_0368*, *PP\_0370*, and *PP\_3554* play a role in valeryl-CoA dehydrogenation, exposing their specificity toward C5 substrates.<sup>[58]</sup>

The two followings steps in the  $\beta$ -oxidation pathway are performed by the FadBA complex. FadB (*PP\_2136*) is endowed with four different enzymatic activities: i) enoyl-CoA hydratase, ii) *cis*- $\Delta^3$ -*trans*- $\Delta^2$ -enoyl-CoA isomerase, iii) 3-hydroxyacyl-CoA dehydrogenase, and iv) 3-hydroxyacyl-CoA epimerase. As such, FadB catalyzes the conversion of 2-*trans*-enoyl-CoA to (*S*)-3-hydroxyacyl-CoA and also its subsequent oxidation to 3-ketoacyl-CoA with the concomitant reduction of NAD<sup>+</sup>.<sup>[55]</sup> The resulting 3-ketoacyl-CoA, still displaying *n* carbon atoms, is converted to the acyl-CoA of *n*–2 carbon atoms while releasing one molecule of acetyl-CoA, ending the first round of the cycle. This reaction is catalyzed by FadA, a 3-ketoacyl-CoA thiolase. At this point, the resulting acyl-CoA<sub>*n*-2</sub> can undergo a new round of  $\beta$ -oxidation, releasing 1 acetyl-CoA per catalytic cycle (Figure 2). Thus, intermediates generated by this pathway will display an aliphatic chain spanning lengths that cover the same number of carbons as the fatty acid used as a substrate to shorter chain lengths produced by the sequential loss of a C2 moiety in each round of  $\beta$ -oxidation.

Several homologs of *fabB* and *fadA* have been described in *P. putida* KT2440. The FadBA complex encoded by *PP\_2136* and *PP\_2137* plays a prevalent role in  $\beta$ -oxidation since its elimination causes a defective pathway that yields polymers with longer chain monomers.<sup>[63]</sup> The FadBAX complex, encoded by *PP\_2214* and *PP\_2215*, may replace the function of the predominant FadBA enzyme complex under certain conditions, as they were observed to be active only when *fadBA* were deleted. Besides, FadBA was shown to be essential for the degradation of *n*-phenylalkanoic acids.<sup>[64]</sup> Furthermore, seven enoyl-CoA hydratases and four HA-CoA dehydrogenases were predicted in *P. putida* KT2440 and their role was also analyzed by global fitness analysis. In line with the findings of previous works, a *fadB* (*PP\_2136*) mutant showed reduced fitness when grown on fatty acids with chain lengths of C6 and longer, confirming that *PP\_2136* encodes the primary enoyl-CoA hydratase/3-hydroxy-CoA dehydrogenase for medium and long fatty acids. By contrast, enoyl-CoA hydratase or HA-CoA dehydrogenase activities for short-chain-length fatty acids seem to be distributed across several enzymes. Growth on hexanoate resulted in moderate fitness defects in mutants disrupted in the predicted enoyl-CoA

hydratases *PP\_2136*, *PP\_2217*, and *PP\_3726*, supporting a role in C6 substrates catabolism. However, functional complementation between *fadB* homologs was observed for the enoyl-CoA hydratase activity toward C5 substrates. In addition, *PP\_2136* (*fadB*), *PP\_2214* (*fadBx*), and *PP\_3755* were shown to encode the hydroxyacyl-CoA dehydrogenase activity toward C6 substrates. This activity was distributed between different enzymes when it comes to the dehydrogenation of 3-hydroxyvaleryl-CoA, with a *PP\_3755* mutant showing only a slight fitness defect on valerate. Lastly, five 3-ketoacyl-CoA thiolases were predicted to be encoded in the genome of *P. putida* KT2440; among them, FadA (*PP\_2137*) and BktB (*PP\_3754*). Both thiolases were observed to act on C7, C6, and C5 substrates.<sup>[58]</sup>

Intermediates of fatty acid degradation displaying different chain lengths should be converted to the corresponding (*R*)-HA monomers to be incorporated into the polymer. Three intermediates from each round of  $\beta$ -oxidation were proposed to be the metabolic link between this fatty acid assimilation pathway and PHA biosynthesis. The most solid evidence establishing a link between  $\beta$ -oxidation and PHA synthesis involves the action of PhaJ, a stereospecific *trans*-enoyl-CoA hydratase, described to convert *trans*-2-enoyl-CoA intermediates into (*R*)-HA-CoAs—the substrate of PhaC (Figure 2). Three variants of this gene, i.e., *phaJ1* (*PP\_4552*), *phaJ3* (*PP\_0580*), and *phaJ4* (*PP\_4817*) have been found in the genome of *P. putida* by homology search against *phaJ1* to 4, the four previously described genes of *P. aeruginosa*.<sup>[54,65]</sup> PhaJ1 showed high specific activity against short- and medium-chain-length enoyl-CoA substrates,<sup>[66]</sup> whereas PhaJ3 and PhaJ4 presented higher affinity for medium- to long-chain-length enoyl-CoA substrates. In addition, *phaJ4* was the only variant overexpressed when cells were grown on fatty acids, indicating that the product of this gene likely plays the most important role in polymer production from fatty acids.<sup>[54,67]</sup> Overproduction of PhaJ in *P. putida* resulted in a positive effect in PHA accumulation, exposing the important role of this enzyme in bridging  $\beta$ -oxidation of PHA-related substrates and polymer accumulation.<sup>[68]</sup>

Two additional (putative) metabolic links between  $\beta$ -oxidation and PHA synthesis were proposed. The first one involves a 3-ketoacyl-CoA reductase that converts 3-ketoacyl-CoA to (*R*)-HA-CoAs. This activity was surmised to be performed by FabG—an enzyme of the FAS pathway that also catalyzes the reverse reaction (Figure 2). In *E. coli* engineered for mcl-PHA production, the FabG enzymes of *E. coli* and *P. aeruginosa* were shown to intercept 3-ketoacyl-CoA intermediates from the  $\beta$ -oxidation pathway to yield (*R*)-HA-CoAs that were subsequently incorporated into PHAs by a plugged-in PHA synthase from a *Pseudomonas* species.<sup>[69]</sup> Despite this key piece of evidence in engineered *E. coli*, the role of FabG as a functional connector of  $\beta$ -oxidation and PHA synthesis in the native *P. putida* context remains unclear. Overexpressing *fabG* was reported to decrease (rather than boost) accumulation of mcl-PHA in *P. putida* KCTC1639—possibly due to the reversible conversion of (*R*)-HA-CoA units into 3-ketoacyl-CoAs.<sup>[68]</sup> Lastly, an epimerase activity mediating the conversion of (*S*)-isomers of HA-CoAs into the (*R*)-form (i.e., the actual substrate for PHA polymerization) has been also assigned to FadB (Figure 2). FadB was proposed as a putative epimerase enzyme based on its identity to the *E. coli* counterpart,<sup>[70]</sup> but its role as such does not seem to be physiologically relevant in *Pseudomonas*



species—at least not under the conditions experimentally tested thus far.<sup>[66]</sup>

## 5. De novo Fatty Acid Synthesis Governs PHA Metabolism from Structurally Nonrelated Substrates

Glycolytic (e.g., sugars) and gluconeogenic (e.g., aromatic compounds, alcohols, and organic acids) carbon sources are considered to be PHA nonrelated substrates, as they are structurally different to the key monomers that make up PHAs. When nonrelated substrates are fed to *P. putida*, carbon fluxes within central catabolic pathways are directed to the key central intermediate acetyl-CoA<sup>[13i,16,71]</sup> and channeled toward PHA formation via FAS (Figure 2). Key evidence supporting this notion is that the composition of polyesters accumulated by *Pseudomonas* is independent of the carbon source when using nonrelated substrates (in contrast to PHA-related substrates). Furthermore, (*R*)-3-hydroxydecanoyl-CoA, the C10 monomer, is one of the main constituents of PHA produced by *Pseudomonas* species from unrelated substrates—suggesting that FAS plays a key role in chain elongation to provide intermediates for mcl-PHA synthesis.

Like virtually all other bacteria, *P. putida* KT2440 has a type II FAS, in which each catalytic activity is contained in a discrete protein (Figure 2). The initiation step of this pathway is the synthesis of malonyl-CoA by acetyl-CoA carboxylation. This reaction is catalyzed by biotin- and ATP-dependent acetyl-CoA carboxylase complex, AccABCD (ACC). In *P. putida* KT2440, ACC is composed of four proteins: i) biotin carboxyl carrier protein, encoded by *accB* (PP\_0559); ii) a biotin carboxylase, encoded by *accC* (PP\_0558), iii) the  $\alpha$  subunit of ACC, encoded by *accA* (PP\_1607), and iv) the  $\beta$  subunit of ACC, encoded by *accD* (PP\_1996). The carboxylation reaction mediated by this enzyme complex takes place in two discrete steps with bicarbonate ( $\text{HCO}_3^-$ ) as the (soluble) carboxyl donor. The first step involves an ATP-dependent transfer of a  $\text{CO}_2$  moiety from  $\text{HCO}_3^-$  to the biotin moiety of AccB, resulting in carboxybiotinoyl-AccB. This reaction is catalyzed by AccC. The second step is the transference of the  $\text{CO}_2$  group from carboxybiotin to acetyl-CoA to form malonyl-CoA in a reaction catalyzed by the two carboxyl-transferase subunits, AccA, and AccD.<sup>[72]</sup>

Malonyl-CoA is then activated via *trans*-acylation to an acyl-carrier protein (ACP) by FabD (PP\_1913). All of the following intermediates in type II FAS are ACP thioesters. In the first round of elongation, the C3 malonyl-ACP is condensed with the C2 acetyl-CoA by FabH (PP\_4379 and PP\_4545) with the concomitant loss of  $\text{CO}_2$ , resulting in acetoacetyl-ACP—the C4  $\beta$ -ketoacyl-ACP. In subsequent rounds,  $\beta$ -ketoacyl-ACP undergoes a reduction catalyzed by the  $\text{NADP}^+$ -dependent  $\beta$ -ketoacyl-ACP reductase, yielding (*R*)-hydroxyacyl-ACP. FabG (PP\_1914) catalyzes this reduction, along with other four putative variants (encoded by *PP\_0581*, *PP\_1852*, *PP\_2540*, and *PP\_2783*). The corresponding (*R*)-hydroxyacyl-ACP is dehydrated by  $\beta$ -ketoacyl-ACP dehydrases, FabA (PP\_4174), and FabZ (PP\_1602), resulting in *trans*-2-enoyl-ACP. This metabolite is finally converted to acyl-ACP by a  $\text{NAD}^+$ -dependent enoyl-ACP reductase. Although four types of bacterial enoyl-ACP reductases (FabI, FabK, FabL, or FabV) have been described so far in bacteria, the gene that encodes this enzyme has not been identified yet

in *P. putida* KT2440. A putative enoyl-ACP reductase (PP\_1852) has been annotated based on the presence of conserved amino acid motifs and homology to other enoyl-ACP reductases.<sup>[72c,73]</sup> A new round of elongation begins with a decarboxylative Claisen condensation between the acyl-ACP of *n* carbon atoms and a new malonyl-ACP residue catalyzed by FabB (PP\_4175) and FabF (PP\_1916 and PP\_3303). This step yields a  $\beta$ -ketoacyl-ACP of *n*+2 carbon atoms, with the concomitant release of ACP and  $\text{CO}_2$ . The condensation thus results in the addition of a C2 moiety in the aliphatic chain of the fatty acid in each round of elongation. The process is repeated until the acyl chain reaches C14 to C18, whereupon the fatty acid is released from the FAS machinery. This pathway is essential, as it provides precursors for cell components (e.g., phospholipids, lipopolysaccharides, and rhamnolipids)—besides its central role in furnishing monomers for PHA synthesis from nonrelated substrates.<sup>[72c,74]</sup>

The metabolic hub sitting between FAS and PHA synthesis is (*R*)-3-hydroxyacyl-ACP, which can be converted to (*R*)-HA-CoAs by the combined activities of PhaG (PP\_1408) and AlkK (PP\_0763). Experimental evidence for this transformation comes from the high levels of transcription detected for *phaG* and *alkK* observed during PHA production when cells grew on nonrelated substrates.<sup>[75]</sup> PhaG has first been described as an (*R*)-3-hydroxyacyl-ACP-CoA transferase, but studies in recombinant *E. coli* revealed that PHA was not produced when either *phaG* or *phaC* were heterologously expressed—indicating that yet another, unidentified enzyme was necessary for channeling fatty acid intermediates to the synthesis of PHA monomers. Furthermore, overexpression of *phaG* in *E. coli* led to the secretion of 3-hydroxydecanoic acid to the extracellular environment (rather than stimulating polymer accumulation). These results pointed out that PhaG could function as a 3-hydroxyacyl-ACP thioesterase, removing ACP from 3-hydroxyacyl-ACP to form free 3-hydroxy fatty acids. Expression of *alkK*, encoding a medium-chain-length fatty acid-CoA ligase, in the same recombinant *E. coli* strain led to PHA production. AlkK converts (*R*)-3-hydroxy fatty acids into (*R*)-HA-CoAs, thus establishing a link to PHA production from nonfatty acid carbon sources.<sup>[76]</sup>

Despite that PHA-related substrates are mostly metabolized via  $\beta$ -oxidation and nonrelated substrates via FAS, these two pathways act coordinately for providing precursors for polymer biosynthesis. Evidence supporting this notion comes from the fact that genes encoding enzymes in the  $\beta$ -oxidation pathway were overexpressed in strain KT2440 when cells were growing on PHA nonrelated substrates, whereas *phaG* was overexpressed when the cells were growing on a fatty acid.<sup>[72a]</sup>

## 6. Regulation of PHA Synthesis in *P. putida* KT2440: From Transcriptional Modulation to Global Signals

The cyclic character of PHA metabolism, where simultaneous biosynthesis and degradation of the polymer occurs in a dynamic and continuous cycle, is a key metabolic feature of *Pseudomonas* species. PHA turnover thus constitutes a *holistic* biochemical system that stores carbon by (de)polymerizing fatty acids intermediates, channeling them from/to central metabolism according to carbon and energy demands. Consequently, the network ruling PHA metabolism consists of a multilevel regulation network



linked to central carbon metabolism<sup>[77]</sup> and driven by *pha* specific and global regulators<sup>[51,78]</sup> (Figure 1).

The presence of five promoters upstream of *phaC1*, *phaZ*, *phaC2*, *phaF*, and *phaI* suggests the existence of polycistronic transcription units. However, the  $P_{C1}$  and  $P_I$  promoters were proposed as the major drivers of *pha* transcription (Figure 1). Gene expression levels of the *pha* cluster responded in a carbon-source dependent manner—higher levels were observed when *P. putida* was grown on PHA-related substrates (e.g., octanoate).<sup>[50]</sup> The *pha* cluster is transcriptionally activated by binding of the transcriptional regulator PhaD to specific operator sequences at the  $P_{C1}$  and  $P_I$  promoters. Even though the effector of PhaD is still unknown, a CoA intermediate of fatty acid metabolism (i.e.,  $\beta$ -oxidation) or the TCA cycle has been proposed to be involved in this regulatory loop.<sup>[50,79]</sup> Additionally, the PhaF phasin has been proposed not only to modulate the expression of the *pha* genes but also to exert an effect on the whole transcriptome—probably due to its DNA binding and histone-like properties, since this protein associates to the nucleoid.<sup>[80]</sup> Although it is still unclear whether or not PhaF specifically binds the promoter region of *pha* genes, deleting *phaF* in *P. putida* KT2442 leads to a significant decrease of *phaC1* and *phaI* transcript levels, thus resulting in a reduction of PHA accumulation.<sup>[22b]</sup> PhaF is a multifunctional phasin that plays a role in granule segregation during cell division and as a transcriptional regulator.<sup>[22b]</sup> Very recently, a direct interaction of PhaF and PhaD has been demonstrated—suggesting the existence of a multicomplex regulatory system formed by PHA granules, a PhaD–PhaF complex, and the target DNA.<sup>[57]</sup>

PHA metabolism has been also suggested to be under the control of the global regulatory systems.<sup>[78a]</sup> For instance, the catabolite repression control protein (Crc), associated with Hfq protein,<sup>[81]</sup> controls PHA synthesis by inhibiting the translation of the *phaC1* mRNA via its interaction with the catabolite motif 5'-AnAA $n$ AA-3' (where  $n$  represents any nucleotide), resulting in a decrease in PHA production.<sup>[82]</sup> Furthermore, the two-component GacS/GacA global antibiotic and cyanide control system was proposed to regulate PHA synthesis in *P. putida* CA-3. GacS/GacA system is part of the Gac/Rsm cascade, which involves interactions with small regulatory RNA molecules, affecting a range of metabolic pathways. GacS is the sensor kinase, and GacA acts as a transcriptional activator of several nonoperonic sRNA genes that sequester a negative regulator of mRNA translation, RsmA, allowing protein synthesis to proceed.<sup>[83]</sup> The translation of the *phaC1* mRNA was observed to be inhibited in a *gacS* mutant by a mechanism yet to be fully understood (Figure 1).

As indicated before, the PHA cycle is closely linked to fatty acid metabolism, since the PhaC synthase substrate, (*R*)-HA-CoA, derives from either  $\beta$ -oxidation or de novo FAS depending on the carbon source used. As such, the different tiers of regulation of these central pathways are expected to affect modulating PHA biosynthesis.<sup>[84]</sup> For instance, the impact of the PsrA regulator, a transcriptional repressor that binds to the promoter region of the *fadBA* operon, on PHA production has been recently studied in *P. putida* KT2440. PsrA was shown to affect the length of the side chain of the monomers according to the activation or repression of components of  $\beta$ -oxidation.<sup>[85]</sup> Additionally, PsrA could affect global regulatory networks by activating the

stationary phase  $\sigma$  factor *rpoS*, as described in *P. putida* and *P. aeruginosa*.<sup>[86]</sup>

PHAs are usually produced under nutrient imbalanced conditions—normally under a carbon excess coupled to a nutrient limitation that results in high C/N or C/P ratios.<sup>[51,87]</sup> High C/N ratios have been considered as a prerequisite for PHA accumulation when PHA nonrelated carbon sources are applied.<sup>[54]</sup> This is probably because 3-hydroxyacyl-ACP-CoA transacylase (PhaG), a key connector of de novo FAS to the PHA cycle, is downregulated under nitrogen excess and also due to the necessity of biomass building-up before PHA accumulation.<sup>[76,88]</sup> Even though nitrogen limitation is not strictly necessary for PHA production from PHA-related substrates, a significant improvement in polymer production can be obtained under these circumstances.<sup>[89]</sup>

Multilevel omics approaches have been performed in *P. putida* KT2440 (and KT2442) for PHA production varying the C/N or C/P ratios using PHA-related or nonrelated substrates in an attempt to shed light on the mechanisms involved behind nutritional imbalances and PHA production. A large number of genes belonging to pathways involved in PHA synthesis were affected depending on the type of limitation, e.g., genes related to amino acid synthesis, nitrogen scavenging, general cellular processes, and transcriptional regulation.<sup>[84,89a,90]</sup> Similarly, *P. putida* cell size depends on the capacity to produce PHA and on the C/N ratio.<sup>[51,87a]</sup> Hence, the influence of the C/N ratio on PHA production can be even more complex than merely checking substrate availability, and the ratio is affected by carbon catabolite repression mechanisms mentioned above. Other regulation mechanisms, apart from the carbon source used and the C/N ratio in the medium, are probably relevant.<sup>[91]</sup> For instance, under low C/N ratios—where PHA degradation is favored—Crc directly inhibits expression of the *pha* cluster, and this effect is suppressed when high C/N ratios are reached<sup>[82,92]</sup> (Figure 1). A subset of PTS proteins, i.e., PtsP, PtsO, and PtsN, has been proposed to be involved in regulating the C/N balance and controlling PHA production in *P. putida*.<sup>[93]</sup> Furthermore, PHA metabolism depends on the overall metabolic status of the cell, which modulates precursor availability.<sup>[94]</sup>  $\beta$ -oxidation activities are regulated differently depending on the NAD(P)H/NAD(P)<sup>+</sup> and acetyl-CoA/CoA ratios, with high ratios fostering polymer biosynthesis.<sup>[78b,95]</sup> The metabolic versatility of *Pseudomonas* can be harnessed to manipulate PHA biosynthesis at different levels of regulation—e.g., by taking advantage of the remarkable degradation capabilities of this species, as indicated in the next section.

## 7. Production of mcl-PHA in *P. putida* from Complex Substrates: Degradation Mechanisms and Conversion of Nonrelated Carbon Sources to PHA Monomers

Upcycling of plastic waste consists of the biotransformation of depolymerized monomers (resulting from chemical transformation or biological degradation) into value-added biochemicals or biopolymers via endogenous or artificial metabolic pathways. In particular, fragments formed after pyrolysis or oxidation of plastic wastes can be employed as carbon sources for PHAs production.<sup>[96]</sup> The native robust catabolic pathways for aromatic



compounds and mcl-PHA biosynthesis in *P. putida* make this species an obvious host for funneling aromatic compounds derived from contaminants and/or recalcitrant substrates into biobased biodegradable polymers.

Petroleum-based polystyrene is the most frequently used plastic globally nowadays. The pyrolysis of this polymer results in the generation of oil composed of styrene [82.8% (w/w)] and low levels of other aromatic compounds. *P. putida* CA-3 can grow on this oil as the sole source of carbon and energy and accumulate mcl-PHA from this complex substrate.<sup>[97]</sup> Additional *P. putida* strains endowed with the ability to grow on styrene as sole carbon source have been isolated,<sup>[44]</sup> e.g., S12, CA-1, and NBUS12 (Table 1). Their natural occurrence suggests that genes encoding catabolic pathways for the degradation of this compound were acquired by horizontal transfer. PHA accumulated from styrene was composed of only aliphatic monomers, ranging from C6 to C10 units. The aliphatic nature of monomers and the length of their chains evidence the cleavage of the aromatic ring and the prevalent role of FAS/PhaG in the generation and capture of PHA monomers. Styrene degradation in strain CA-3 involves an upper pathway that converts styrene into phenylacetate and an independently regulated lower pathway initiated via activation of phenylacetate to phenylacetyl-CoA. Phenylacetate is an intermediate in the degradation of other aromatic compounds, and *P. putida* CA-3 and S12 are also able to use it as the sole carbon and energy source.<sup>[44a]</sup> This intermediate is further catabolized by a dedicated phenylacetate degradation pathway that involves oxidation of the aromatic core of the molecule, ring cleavage, and  $\beta$ -oxidation cycling of the alicyclic compound, ultimately leading to acetyl-CoA. This pathway is also present in *P. putida* KT2440 and is encoded by the *paa* genes. Acetyl-CoA is further metabolized via FAS/PhaG to provide monomers for PHA synthesis<sup>[98]</sup> (Figure 1).

Another extensively used oil-derived plastic is polyethylene terephthalate (PET). *P. putida* GO16, a strain isolated from a PET recycling plant, can grow and produce aliphatic mcl-PHA from terephthalate, a product of PET pyrolysis. Terephthalate is catabolized via the central intermediate protocatechuate through the  $\beta$ -keto adipate pathway, implying breakage of the aromatic ring and formation of acetyl-CoA—again, channeled to PHA monomer synthesis via FAS/PhaG. Because of this, PHA accumulated by terephthalate-grown strain GO16 was composed mainly of 3-hydroxydecanoate. Cofeeding with terephthalate and glycerol from biodiesel manufacture waste (containing >C18 fatty acids) also led to the production of mcl-PHA with 3-hydroxydecanoic acid as predominant monomer—but 3-hydroxytetradecanoic and 3-hydroxytetradecenoic acid were also present, probably resulting from the catabolism of C18 fatty acids.<sup>[42]</sup> Recently, the terephthalate-degrading *P. putida* GO16 strain has evolved to be able to use ethylene glycol (another monomer from PET hydrolysis) as a substrate for the production of PHA. The evolved strain, termed *P. putida* GO16 KS3, produced 0.15 g L<sup>-1</sup> PHA [7% (w/w)] when fed with both terephthalate and ethylene glycol.<sup>[99]</sup>

Lignocellulose is the most abundant biopolymer available on earth as waste biomass and its use as a resource for sustainable fuels and chemical production leads to the generation of substantial quantities of lignin by-products that can be used as feedstocks for other biobased processes.<sup>[95b,100]</sup> *P. putida* has proven to be an efficient cell factory for lignin valorization.

Lignin is a highly cross-linked phenolic polymer derived from vegetal biomass and a very difficult-to-degrade substrate; breaking down this material qualifies as harsh biotransformation as it has to be executed in the presence of a *plethora* of inhibitory compounds and highly reactive intermediates. The intrinsic heterogeneity of lignin yields complex mixtures of aromatic compounds after pretreatment, depolymerization, and hydrolysis. The degradation products are usually aromatic building blocks, e.g., *p*-coumaric acid, vanillic acid, and ferulic acid in addition to the already-described aromatic products from the degradation of oil-derived plastics styrene and phenylacetic acid. Many lignin-derived aromatic compounds are catabolized by the  $\beta$ -keto adipate pathway, where central intermediates like catechol and protocatechuate are generated. From these intermediates, dioxygenase enzymes cleave C–C bonds in the aromatic rings to yield ring-open species that are oxidized via central carbon catabolic pathways to acetyl-CoA that is then channeled to mcl-PHA production via FAS/PhaG (Figure 2). In *P. putida* KT2440, enzymes from the  $\beta$ -keto adipate pathway are encoded by the *pca* genes. A recent example exploiting this feature indicated that *P. putida* KT2440 accumulates  $\approx$ 32% (w/w) mcl-PHA when grown in a lignin-enriched substrate (corn stover alkaline pretreated liquor), containing mainly *p*-coumaric acid, vanillic acid, ferulic acid, and acetate as carbon sources. Besides producing polymer out this complex mixture, *P. putida* was also able to accumulate mcl-PHA from *p*-coumaric, ferulic acid, and acetate as sole carbon sources, resulting in equivalent PHA contents of 34%, 39%, and 20% (w/w), respectively.<sup>[101]</sup>

Another lignin-utilizing *P. putida* strain, A514, was shown to grow and accumulate aliphatic PHAs when using vanillic acid as the sole carbon source. In this strain, the transcription of *phaJ4*, *phaG*, and *alkK* is highly induced under nitrogen starvation conditions—yet only low amounts of PHA were accumulated. High levels of *phaG* and *alkK* expression were likewise observed under high nitrogen conditions, suggesting that PhaG and AlkK not only have a role in PHA biosynthesis but they are also essential for cell growth on lignin derivatives.<sup>[102]</sup>

Strain KT2440 was also able to produce aliphatic PHA from benzoate, a lignin model compound that is used for the study of bacterial catabolism of aromatics. A different monomer distribution was observed when cells were grown in a medium with N limitation compared to N surplus. When N was limited, C16, C12, C11, C10, C8, and C6 units were incorporated into the polymer, whereas C16 and C10 monomers were incorporated in the presence of N surplus—suggesting that N availability is a key factor regulating the benzoate degradation-to-PHA biosynthesis circuit. Also, this strain can synthesize 20% (w/w) PHA from acetate, a substrate that is directly converted to acetyl-CoA by an acetyl-CoA synthase encoded by *acsAI-II* (PP\_4487 and PP\_4702) followed by its transformation to PHA monomers via FAS/PhaG<sup>[101,103]</sup> (Figure 2).

Although these examples show that *P. putida* is suitable for the upcycling of recalcitrant polymers, many issues still need to be overcome for efficient PHA production from these carbon sources. In particular, these substrates are toxic even at low concentrations and traditional PHA production approaches (i.e., unbalanced C/N ratios) are impractical, requiring other strategies to boost polymer synthesis as explained in the sections below.



## 8. Expanding the Natural Diversity of PHAs in *Pseudomonas*: Incorporation of Functional Chemical Groups in PHAs by Feeding Approaches Using Modified, PHA-Related Substrates

Material properties of PHA are highly dependent on monomer composition, the length of the PHA side chain and its chemical nature have a significant influence on the properties of the bioplastic, e.g.,  $T_g$ ,  $T_m$ , and mechanical properties like the level of stiffness or flexibility. Because of this, the introduction of new elements and chemical groups of different nature into the polymer structure are of great importance for rendering the synthesis of novel polyesters with diversified and improved properties to meet the demand for industrial and medical applications.

The rich native metabolism of *Pseudomonas*, coupled to the broad substrate specificity of PHA synthases in these species, offers a phenomenal playground for the incorporation of a wide variety of monomers in the polyesters. Modified monomers introduced by *P. putida* strains in PHAs for the production of novel polymers and their emerging properties are summarized in Figures 3–5. Besides the use of PHA-related substrates (Figure 3A), early examples of cofeeding include the production of methyl-branched PHAs by incorporation of methyl groups in the PHA accumulated by *P. putida* Gp01 (formerly known as *P. oleovorans*) grown in the presence of 6-methylnonanoic, 7-methylnonanoic, 8-methylnonanoic or 9-methyldecanoic acid as an additive to the culture medium.<sup>[104]</sup>

Even-branched monomers, containing side groups that vary significantly in size and polarity from the canonical *n*-alkanoate structure, can be captured into the polymer architecture. Aromatic monomers, encompassing phenyl, phenoxy, cyanophenoxy, nitrophenoxy or thiophenoxy groups; units containing thioester, thioether, and sulfur groups; and precursors displaying a variable number of halogens atoms are among chemical modifications that can be added to PHAs.<sup>[105]</sup> All these substituents were introduced in the side chains of PHA by feeding with modified alkanic acids bearing the desired chemical groups. The use of these substrates as single carbon sources and PHA precursors, however, usually had detrimental effects on both bacterial growth and polymer accumulation—which translated into low incorporation of novel chemical groups into the polyesters. Because of this occurrence, most of the functional groups were introduced into PHA by sequential feeding or cofeeding approaches with alkanic acids, thereby leading to polymers with mixed monomer composition.

### 8.1. Biosynthesis of PHAs Containing Aromatic Monomers and Fluorinated, Sulfonated, or Nitrogenated Derivatives

#### 8.1.1. Incorporation of Phenyl and Methylphenyl Groups

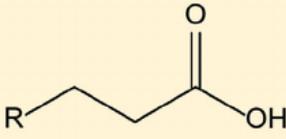
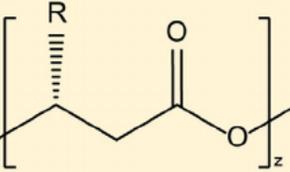
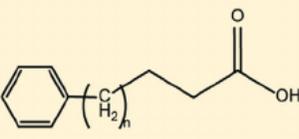
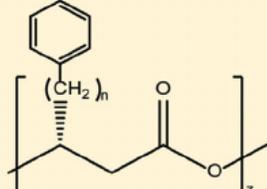
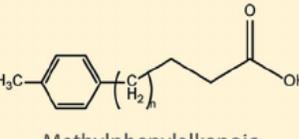
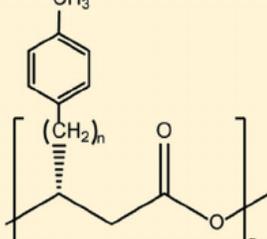
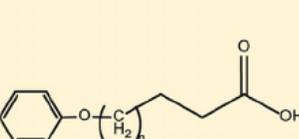
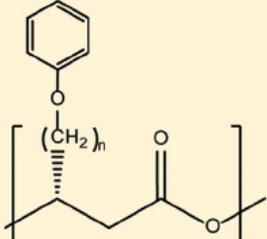
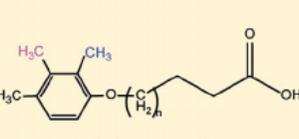
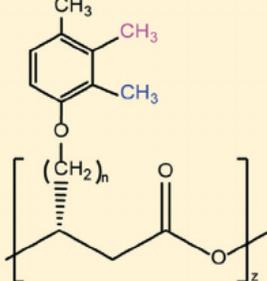
As indicated in the previous section, polystyrene is a vinyl polymer with a long hydrocarbon chain forming the backbone and bulky phenyl groups as pendant groups. This structure gives polystyrene thermoplastic properties.<sup>[106]</sup> Taking inspiration on this physicochemical feature, several  $\omega$ -phenylalkanoic acids, displaying C5 to C11 units in the aliphatic chain, have been used as the sole carbon source or as cofeeding substrates for

the production of PHAs containing aromatic pendant groups or poly(3-hydroxyphenylalkanoates) in different *P. putida* strains (Figure 3B). Poly(3-hydroxyphenylalkanoates) produced from 6-phenylhexanoic acid by *P. putida* KT2440 (which, incidentally, proved to be the most effective strain for the accumulation of such polymers) contained mostly 3-hydroxy-6-phenylhexanoate (C6) units. Interestingly, when the molecules used as carbon sources were  $\omega$ -phenylalkanoic acids containing an odd number of carbon atoms (7-phenylheptanoic, 9-phenylnonanoic, or 11-phenylundecanoic acid), 3-hydroxy-5-phenylvalerate (C5) was the major monomer. Under these conditions, the polymer yield ranged from 0.08 g L<sup>-1</sup> for 7-phenylheptanoic acid and 0.61 g L<sup>-1</sup> from phenylhexanoate [with PHA contents of 24% and 47% (w/w), respectively]. Cofeeding of most of these substrates with nonanoic acid gave rise to PHAs with mixed monomer composition [polymer accumulation reached up to 52% (w/w), with a PHA concentration of 0.6 g L<sup>-1</sup>, when using 9-phenylnonanoic acid]. The resulting polyesters contained monomers with both phenylalkyl substituent groups (between 40% and 78% mol/mol) and aliphatic units.<sup>[107]</sup> Besides, when *P. putida* KT2440 was fed with butyric acid as a carbon source and 5-phenylvalerate as a substrate for PHA synthesis, this strain produced the homopolymer poly(3-hydroxy-5-phenylvalerate), growing to 1.9 g L<sup>-1</sup> of biomass with a PHA content of 58% (w/w). Growth on butyric acid and 6-phenylhexanoate, however, resulted in a copolymer of 10.1% (mol/mol) 3-hydroxy-4-phenylbutyrate and 89.4% (mol/mol) 3-hydroxy-6-phenylhexanoate accumulated in the cells (1.4 g L<sup>-1</sup> of total biomass) at 21% (w/w). Both polymers were amorphous, with  $T_g$ s of 15.7 and 5.3 °C, respectively.<sup>[108]</sup>

Similar results were observed in *P. putida* U when grown with  $\omega$ -phenylalkanoic acids ranging from C5 to C10 ( $15 \times 10^{-3}$  M) as a sole carbon source—this strain accumulated from 10% to 28% (w/w) of polymer containing phenyl groups (Figure 3B). When this strain was cultured using 6-phenylhexanoic acid as a carbon source, a homopolymer of 3-hydroxy-6-phenylhexanoate was produced—whereas copolymers were formed when the carbon source was 8-phenyloctanoic or 10-phenyldecanoic acid. In the case of 8-phenyloctanoic acid, the polymer contained two different monomers: 3-hydroxy-6-phenylhexanoate and 3-hydroxy-8-phenyloctanoate at relative proportions of 62.5% and 37.5% (mol/mol), respectively. However, when the carbon source was 10-phenyldecanoic acid, the polymer contained units of 3-hydroxy-6-phenylhexanoate (48.2%), 3-hydroxy-8-phenyloctanoate (31.2%), and 3-hydroxy-10-phenyldecanoate (20.6%), respectively. By contrast, as observed for *P. putida* KT2440, when the carbon sources were  $\omega$ -phenylalkanoic acids containing an odd number of carbon atoms, the only bioplastic produced was a homopolymer of 3-hydroxyphenylvalerate.<sup>[109]</sup>

The monomer composition observed for the poly(3-hydroxyphenylalkanoates) produced in these strains indicated that phenylalkanoic acids were catabolized by the  $\beta$ -oxidation pathway and that the polymerases responsible for the condensation of aromatic monomers into polymers recognize CoA derivatives of 3-hydroxyalkanoic acids containing acyl chains longer than four carbon atoms as substrates. Furthermore, production of poly(3-hydroxyphenylalkanoates) in *P. putida* species expanded the current knowledge on substrate versatility of the  $\beta$ -oxidation pathway, as the enzymes involved in transport, activation to CoA-thioesters, and polymerization of the



A PHA-related substrates	Conventional mcl-PHA	General properties	Reference polymer		
 <p>Alkanolic acids</p>		Semi-crystalline polymers with high flexibility and high elongation at break values. Relatively low mechanical strength ( $\sigma^t$ ), temperature of melting ( $T_m$ ) and glass transition temperature ( $T_g$ ).	P(3HHx-co-HO-co-HD-co-HDD-co-HTD)  $T_m = 58.1$ to $66.8^\circ\text{C}$ and $T_g = -42$ to $-40^\circ\text{C}$		
B Modified PHA-related substrates used for feeding	Novel mcl-PHA	Emerging properties	Production parameters range		
Organic aromatic alkanolic acids					
 <p>Phenylalkanoic acids</p>		High content of C5 or C6 monomers (semi-crystalline polymers); longer alkyl chain (amorphous polymers). Low crystallinity due to long alkyl chain length in the aromatic side group leads to decreased $T_g$ values.	PHA concn. (g/L) 0.1 – 1.5	PHA % (w/w) 10 – 59	Total biomass (g/L) 0.3 – 1.9
 <p>Methylphenylalkanoic acids</p>		C5 monomers result in a crystalline polymer.	0.04 – 0.35	8 – 31	0.5 – 1.1
Alkanolic acids containing phenoxy groups					
 <p>Phenoxyalkanoic acids</p>		Introduction of aromatic side chains prevents crystallization.	0.46 – 1.9	23 – 47	2 – 3.9
 <p>Methylphenoxyalkanoic acids</p> <p>CH<sub>3</sub> in either <i>ortho</i>, <i>meta</i> or <i>para</i> position of the aromatic ring</p>		Altered material properties depending on the CH <sub>3</sub> substituent position. PHA containing <i>m</i> -methylphenoxy (C4 and C6) monomers was crystalline, white and brittle.	0.028 – 0.085  0.009  0.002	<i>para</i> 13  <i>meta</i> 3  <i>ortho</i> 0.8	0.22 – 0.65  0.31  0.23

**Figure 3.** A) Structure and properties of traditional PHAs. B) Structure, properties, and production parameters of novel PHAs obtained by feeding with organic aromatic alkanolic acids and alkanolic acids containing phenoxy groups. Concn., concentration.



monomers were found to be the same for phenylalkanoic acids as for alkanolic acids.<sup>[110]</sup>

Physicochemical characterization of these novel PHAs was performed in *P. putida* U disrupted in the gene that encodes the 3-ketoacyl-CoA thiolase (*fadA*) cultured in a medium containing different aromatic fatty acids (6-phenylhexanoic, 7-phenylheptanoic, a mixture of them, or 8-phenyloctanoic acid) as carbon sources and 4-hydroxyphenylacetic acid (which cannot be directly used for polymer synthesis, but efficiently supports bacterial growth). Studies on the chemical structure of the copolymers produced when using the phenyl-C7 or C8 substrates (or mixtures thereof), revealed that they were true copolymers but not a mixture of homopolymers and that the different monomeric units were randomly incorporated in the macromolecular chains. Microstructural analysis by NMR spectroscopy revealed that there was a random distribution of the  $\omega$ -phenyl macromolecular chains, independent of the feed medium. The high-molecular-weight polymer obtained was shown to be amorphous, which was consistent with the presence of an intrinsic random distribution of monomer units. Poly(3-hydroxyphenylhexanoate) exhibited a prominent  $T_g$  at  $-1.3$  °C, a value lower than the reported for the homopolymer having one C unit less, poly(3-hydroxyphenylvalerate), which presented a  $T_g$  of  $13$  °C. The copolymer containing C6 and C8 units had a  $T_g$  of  $-14.8$  °C due to the presence of a fraction of longer side alkyl chains, whereas the copolymer containing C5 and C7 units showed a  $T_g$  at  $-11.2$  °C and the copolymer presenting C6, C7, and C8 units displayed this transition at  $-8.2$  °C.<sup>[109]</sup>

*P. putida* CA-3 was also shown to accumulate PHA heteropolymers composed of aromatic and aliphatic monomers from C5, C6, C7, C8, and C10 phenylalkanoic acids [45% to 59% (w/w), corresponding to  $0.48$  to  $0.86$  g L<sup>-1</sup> of PHA] (Figure 3B). The polymers presented a 98% content of aromatic monomers and (*R*)-3-hydroxyphenylvalerate or (*R*)-3-hydroxyphenylhexanoate were the most abundant monomers found in polymers accumulated from phenylalkanoic acids with an uneven and even number of carbons on the acyl side chain, respectively. The PHAs accumulated from phenylvaleric and phenylhexanoic acid were partially crystalline with  $T_m$  values of  $51.5$  and  $52.1$  °C, respectively. The polymers accumulated from phenylheptanoic, phenyloctanoic, and phenyldecanoic acid had no  $T_m$ —indicating that these polymers were amorphous. The  $T_g$  of the polymers varied between  $13.2$  °C for PHA accumulated from phenylvaleric acid to  $-14.3$  °C for PHA produced from phenyloctanoic acid. Poly(3-hydroxyphenylalkanoates) accumulated by *P. putida* CA-3 had low crystallinity compared to aliphatic PHA polymers and their properties varied significantly depending on the substrate, becoming increasingly glue-like as the acyl chain length of the growth substrate was longer.<sup>[97,111]</sup> Taken together, the results of these works conclude that  $T_g$  values decrease with longer alkyl side chains and that was attributed to the increasing molecular flexibility of the monomers containing phenyl groups and alkyl side chains longer than three methyl groups. This type of PHA copolymer was proposed to be suitable for designing controlled delivery systems and for preparing composites with biomedical applications.<sup>[112]</sup>

Tolylalkanoates (*p*-methylphenylalkanoates) were also tested as substrates for aromatic PHA production (Figure 3B). *P. putida* GP01 grown on 5-*p*-tolylvaleric acid produced poly(3-hydroxy-5-

*p*-tolylvalerate). The polymer produced was the first example of a crystalline aromatic-containing PHA, with  $T_g$  of  $18$  °C and  $T_m$  of  $95$  °C. Also, when *P. putida* was fed with 9-*p*-tolylnonanoic acid, very low amounts [ $0.04$  g L<sup>-1</sup>, 8% (w/w)] of a polymer comprising 3-hydroxy-5-*p*-tolylvalerate units were produced. When cofed with nonanoic acid, the amount of polymer increased to  $0.35$  g L<sup>-1</sup> and 31% (w/w) and it was composed of 10% (w/w) 3-hydroxy-5-*p*-tolylvalerate monomers.<sup>[107]</sup> In contrast to poly(3-hydroxy-5-phenylvalerate), an amorphous polymer, poly(3-hydroxy-5-*p*-tolylvalerate) was described to be a crystalline material. This observation indicates that the presence of a methyl group at the *para* position of the phenyl ring allows the polymer chains to form a spatially ordered structure.<sup>[113]</sup>

The chemical and physical properties of aromatic PHAs are different from those of aliphatic PHAs, and they vary widely depending on the types of aromatic monomers and aliphatic carbon chain length (Figure 3). In general,  $T_g$  increased with the aromatic monomer content, and the crystallization of PHA was strongly inhibited with the introduction of even a small amount of aromatic monomers. This behavior was attributed to the rigidity of the aromatic rings in the side groups as the bulky size of the side group of alkanoyl-CoA limits its incorporation into the polymer and longer chains of methylene groups between the modifying chemical group and the carbonyl group favor PHA accumulation. Additionally, the degradation rate of aromatic PHAs seems to be slower than that of *mcl*-PHAs—which poses a question about their environmental fate once disposed of.

### 8.1.2. Phenoxy and Methylphenoxy Groups

Commercial polymers containing phenoxy groups, or phenoxy resins, present high adhesive strength, flexibility, and toughness as well as outstanding chemical and heat resistance. Because of these properties, they are often used in epoxy and phenolic resin products including coatings, composites, adhesives, and inks. Monomers containing phenoxy groups in the terminal carbon of the aliphatic chain can also be incorporated into PHAs. *P. putida* BM01 produced aromatic copolyesters when grown with 11-phenoxyundecanoic acid, a modified C11 unit, as the sole carbon source.<sup>[113b]</sup> The polymer concentration and content were  $0.46$  g L<sup>-1</sup> and 23% (w/w), respectively, and it was composed of two monomer units: 3-hydroxy-5-phenoxyvalerate as the major component (72% to 85% mol/mol) and 3-hydroxy-7-phenoxyheptanoate as the minor component (15% to 28% mol/mol). Despite the presence of two monomer units with different lengths of side-chain spacers, the copolymer produced had a  $T_g$  of  $70$  °C and a sharp glass transition between  $9.4$  and  $18.8$  °C.

When the same bacterium was cofed with 11-phenoxyundecanoic acid and octanoate in different proportions, the yield of polymer increased up to  $1.85$  g L<sup>-1</sup> and 47% (w/w), and a random copolyester composed of aromatic and aliphatic monomers [poly(3-hydroxy-5-phenoxyvalerate-*co*-3-hydroxy-7-phenoxyheptanoate-*co*-3-hydroxy-9-phenoxyundecanoate-*co*-3-hydroxyalkanoate)] was synthesized as a result. The increased conversion yield when octanoate is present in the medium implies a limited utilization of 11-phenoxyundecanoic acid for cell growth, coupled with an enhanced channeling of the substrate to



the polyester formation. An increase in 11-phenoxyundecanoic acid concentration in the cofeeding mix changed the ratio between aromatic monomers, shifting to 3-hydroxy-7-phenoxyheptanoate as the major monomer unit and leading to the presence of the longer 3-hydroxy-9-phenoxyundecanoate unit. This situation could be due to the easy assimilation of octanoate as a carbon source for cell growth, which limits the degradation of 3-hydroxy-7-phenoxyheptanoate to provide biomass precursors via  $\beta$ -oxidation. Along the same line of reasoning, when butyric acid was added to an 11-phenoxyundecanoic acid-containing medium, *P. putida* BM01 produced a copolyester composed of only aromatic monomer units with a significant increase in the content of 3-hydroxy-7-phenoxyheptanoate, as C4 units are now used for biomass formation but are not incorporated into the polymer. The  $T_m$  of these polyesters decreased from 54 to 45 °C as the aromatic monomer content increased from 0 to 45% (mol/mol)—illustrating how the random introduction of aromatic side chains into PHAs prevents crystallization.<sup>[113b]</sup>

*P. putida* KCTC2407 was also reported to produce PHAs containing methylphenoxy groups when grown with 6-*p*-methylphenoxyhexanoate or 8-*p*-methylphenoxyoctanoate. When using these substrates as the sole carbon source, cells produced 0.028 g L<sup>-1</sup> [i.e., 12.5% (w/w)] and 0.085 g L<sup>-1</sup> [i.e., 13% (w/w)], respectively, of a PHA bearing the *p*-methylphenoxy substituent. The copolyester consisted of 3-hydroxy-6-*p*-methylphenoxyhexanoate and 3-hydroxy-4-*p*-methylphenoxybutyrate as the major and minor constituents, respectively. When this *P. putida* strain was cultivated on various molar mixtures of 8-*p*-methylphenoxyoctanoate and nonanoate, the mixture of polymers synthesized had such a richness in the structural composition that the monomers could be separated by extraction with *n*-hexane according to the hydrophobicity introduced by the aromatic monomers. This strain could also synthesize a small amount of PHA from 8-*m*-methylphenoxyoctanoate bearing *m*-methylphenoxy groups [ $\approx 9$  mg L<sup>-1</sup>, corresponding to  $\approx 3\%$  (w/w)]. Upon purification, the resulting material was a white, brittle material consisting of 3-hydroxy-6-*m*-methylphenoxyhexanoate and 3-hydroxy-4-*m*-methylphenoxybutyrate units. A small amount of polymer ( $\approx 2$  mg L<sup>-1</sup>) was also produced when 8-*o*-methylphenoxyoctanoate was used as the sole carbon source. However, this PHA did not contain any corresponding functional groups in the side chain. Although the physical properties of these polymers were not analyzed in this work, the results indicate that biosynthesis of PHAs bearing methylphenoxy substituents from methylphenoxyoctanoates (and their material properties) are highly dependent upon the methyl substituent position.<sup>[47]</sup>

### 8.1.3. Incorporation of Nitrogen-Containing Monomers

Incorporation of cyano ( $-\text{C}\equiv\text{N}$ ) and nitro ( $-\text{NO}_2$ )-phenoxy side-chain substituents into PHA was tested in *P. putida* KT2442 and *P. putida* GPo1 (Figure 4). *P. putida* KT2442 was cultivated in a first, cell-growing step on citrate, and then in a second, polymer-producing stage in mixtures of octanoate with either *p*-cyanophenoxyhexanoate, *p*-cyanophenoxyvalerate, *p*-cyanophenoxybutyrate, or *p*-nitrophenoxyhexanoate—all of

them added as substrates for polymer accumulation. When fed with octanoate and cyanophenoxy-alkanoic acids, this strain produced  $\approx 0.08$  to 0.27 g L<sup>-1</sup> of structurally heterogeneous polymers after 1 day of the second fermentation stage, having highly variable contents of cyanophenoxy-containing units in different chains and chain segments. When fed with octanoate and *para*-nitrophenoxyhexanoate, lower amounts of polymer ( $\approx 0.05$  g L<sup>-1</sup>) were produced. Thus, higher polymer titers were achieved when using *para*-cyano- as opposed to *para*-nitro-substituted phenoxyalkanoate carbon sources.

*P. putida* strongly preferred incorporation of *p*-cyanophenoxyhexanoate, and the PHA produced from mixtures of such modified carbon source and octanoate presented a 24% (mol/mol) fraction of 3-hydroxy-6-*p*-cyanophenoxyhexanoate side groups. Importantly, lower amounts of polymer were produced when substrates containing C4 or C5 units in the aliphatic chain were used as the primary carbon source. This occurrence would indicate that *P. putida* was almost incapable of metabolizing these substrates to cyanophenoxy-containing PHA repeat units with substrates containing less than a three-methylene spacer group between the cyanophenoxy substituent and the 3-position of the alkanolic acid. An increase in the content of cyanophenoxy groups resulted in a reduction of the  $T_m$ , more rapid crystallization, and an additional  $T_g$  transition.<sup>[114]</sup>

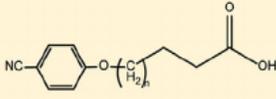
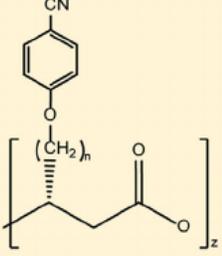
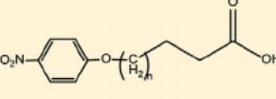
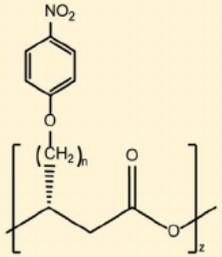
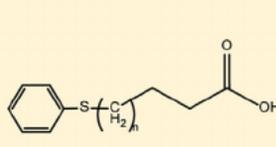
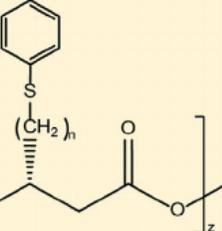
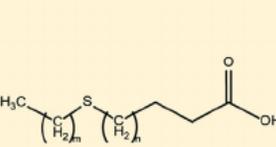
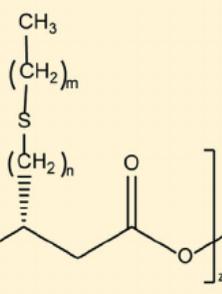
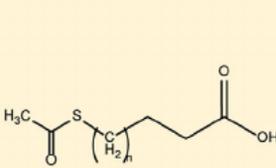
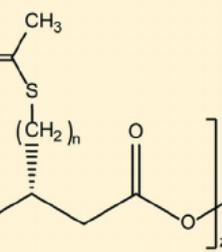
Polymers containing nitrophenyl groups were isolated from *P. putida* GPo1 when grown with variable ratios of 5-(2',4'-dinitrophenyl)valeric acid and nonanoic acid. PHAs had 1.2–6.9% (mol/mol) of repeating units containing 4'-nitro and/or 2',4'-nitrophenyl rings, depending on the molar ratio and total concentration of the two carbon sources supplied. Thermal analysis of the polymers produced indicated the presence of two  $T_g$  values,  $T_{g1} = -35.95$  °C and  $T_{g2} = 28.74$  °C, and a single  $T_m$  value of 56.42 °C. Since no new  $T_m$  value was observed, these data suggest the coexistence of two polymer fractions, one having nitrophenyl groups and the other that is a copolymer with nonanoic acid. The polymer containing nitrophenyl units is amorphous, thus lacking crystalline domains. Despite that this new material has only 4–7% (mol/mol) nitrophenoxy units, the physical appearance (yellow and elastic) was different from that of the polymer obtained from nonanoic acid (whitish and sticky).<sup>[115]</sup>

### 8.1.4. Introduction of Sulfonated Monomers

Studies performed in *P. putida* 27N01 using 11-thiophenoxyundecanoic acid as substrate showed that thiophenoxy groups can also be incorporated into the polymer—with an accumulation of 0.135 g L<sup>-1</sup> PHA [i.e., 19.5% (w/w)] with 3-hydroxy-5-thiophenoxy-pentanoate as the primary monomer unit and 3-hydroxy-7-thiophenoxyheptanoate as a minor unit (Figure 4). The monomer distribution is similar to that observed when using 11-phenoxyundecanoic acid as substrate, surmising that substrates with modified monomers are similarly processed via  $\beta$ -oxidation. This biopolyester was an amorphous elastic polymer with a  $T_g$  of 4 °C.<sup>[39]</sup>

The introduction of thioester or thiol terminal groups in biopolymers allows for further polymer modifications because these groups can easily undergo transesterifications reactions via “click” chemistry. The reactivity of these sulfur-containing



Modified PHA-related substrates used for feeding	Novel mcl-PHA	Emerging properties	Production parameters range		
Nitrogen-containing phenoxyalkanoic acids					
 <p>Cyanophenoxyalkanoic acids</p>		An increase in the content of monomers containing cyanophenoxy groups resulted in a reduction of $T_m$ , rapid crystallization and an additional $T_g$ transition.	PHA concn. (g/L)	PHA % (w/w)	Total biomass (g/L)
			ca. 0.08 – 0.27	NR	NR
 <p>Nitrophenoxyalkanoic acids</p>		Amorphous polymers.	0.5	NR	NR
Sulfur-containing alkanolic acids					
 <p>Thiophenoxyalkanoic acids</p>		Amorphous elastic polymers.	0.135	19.5	0.7
 <p>Alkylthioalkanoic acids</p>		Thioether groups can undergo transesterifications reactions. Light-yellow, translucent and very glutinous polymers.	NR	NR	NR
 <p>Acetylthioalkanoic acids</p>		Thioester groups can undergo transesterifications reactions. Amorphous polymers. $T_g$ decreases with increasing content in alkyl monomers, and increases with the presence of thioester groups. Good thermal stability (up to 200°C).	0.22	18.6	1.2

**Figure 4.** Structure, properties, and production parameters of novel PHAs obtained by feeding of *P. putida* cultures with nitrogen- and sulfur-containing alkanolic acids. Concn., concentration; NR, not reported.



groups, generated by thioester hydrolysis, opens up a wide range of applications for novel thio-functional biopolymers (e.g., oxidation of the sulfur atom with iodide would lead to PHAs with sulfonated side chains). On this background, the synthesis of PHAs containing thioether groups in the side chain has been explored. These polymers were identified in the quest for new materials with antifungal and antibacterial properties for medical applications. Both engineered strains of *C. necator*, harboring the PHA synthase from *P. mendocina*, and *P. putida* KT2440 were fed with chemically-synthesized alkylthioalkanoic acids. *P. putida* KT2440 produced a polymer containing thioether functional groups when cofed with propylthioundecanoic acid and nonanoic acid. Besides the monomers produced from the alkyl substrate, significant amounts of different sulfur-containing constituents [sulfur content of 6% (w/w)] were found in PHA—but their composition could not be determined. The polymers were described to be light-yellow, translucent, and very glutinous, with a typical thiol scent.<sup>[116]</sup>

Two strains of *P. putida* KT2442, wild-type, and its  $\Delta$ *fadB* derivative were tested for the generation of novel PHAs containing thioether groups in the side chain (termed PHACOS). When cofed with decanoic acid (both as growth substrate and inducer of polymer synthesis) and 6-acetylthiohexanoic acid as the PHACOS precursor, these strains accumulated 0.22 g L<sup>-1</sup> of a polymer containing both nonfunctionalized monomers (C10, C8, and C6 units) and the 6-acetylthiohexanoate—related monomers, 6-acetylthio-3-hydroxyhexanoate and 4-acetylthio-3-hydroxybutanoate. The proportion of each monomer was quite similar to the ratio of precursors in the culture medium. As indicated in the previous sections, some reports indicated that the molar ratio of functional groups in the mixture of substrates is largely maintained in the final polyester.<sup>[117]</sup> When compared to the wild-type strain, the *fadB* mutant strain accumulated PHACOS with a low content of thioester-based monomers and with alkyl monomers exhibiting long side chains—resulting in high molecular mobility, enabling the polymers chains to crystallize at a very slow rate.

PHACOS polymers display an amorphous behavior due to the inability of the polymer backbone to organize in a crystal structure. This property is connected to the presence of the bulky side chains and functional groups, resulting in stiffer and less mobile chains. In addition, PHACOS showed good thermal stability up to 200 °C, expected to confer superior processing properties and potentially interesting mechanical features. Amorphous mcl-PHA, with low  $T_g$  values, is predicted to display relatively high softness and elasticity, two properties of interest in materials for biomedical applications.<sup>[118]</sup> In particular, the  $T_g$  values of PHACOS are influenced by two factors.  $T_g$  decreases with increasing content in alkyl monomers (according to the length of the PHA side chains), and it increases with the presence of thioether groups (due to the decreased chain mobility that the thioether moiety confers).

PHACOS were shown to display antibacterial properties, as these polymers efficiently inhibit the growth of methicillin-resistant *Staphylococcus aureus* both in vitro and in vivo. This ability has been ascribed to the functionalized side chains containing thioether groups that support a contact active surface mode of antibacterial action. Because of this, PHACOS were proposed as an infection-resistant biomaterial.<sup>[119]</sup>

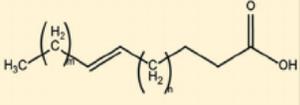
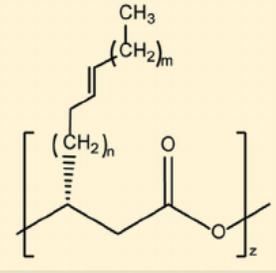
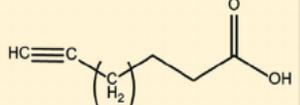
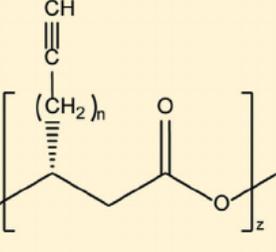
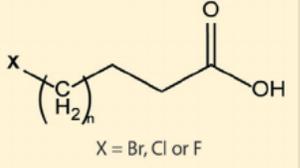
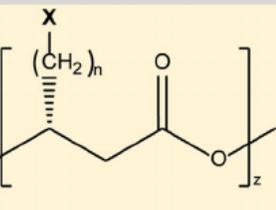
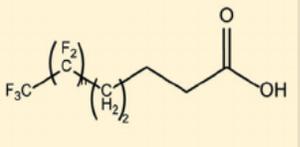
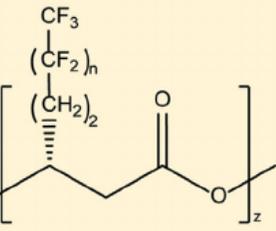
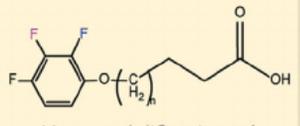
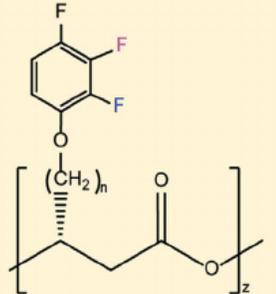
### 8.1.5. Unsaturated PHAs: Monomers Containing C=C or C≡C Bonds

Polymers bearing reactive groups such as C=C or C≡C bonds have attracted much attention because they can be chemically modified by several methods, e.g., electron beam irradiation,  $\gamma$  irradiation, and epoxidation to generate novel materials with improved mechanical properties. PHAs isolated from *P. putida* KT2442 cultivated on the unsaturated long-chain fatty acids oleic acid (i.e., *cis*-9-octadecenoic acid, C18:1) and linoleic acid (i.e., *cis,cis*-9,12-octadecadienoic acid, C18:2) incorporated saturated and unsaturated mcl-3-hydroxy fatty acids as constituents. The PHA content was in the range of 15–35% (w/w) for both carbon sources (Figure 5).

Processing of unsaturated fatty acids with C=C bonds extending from odd-numbered carbon atoms via  $\beta$ -oxidation results in intermediates with a *cis*- or *trans*-double bond at position C3, which cannot be further processed. A (3-*cis*→2-*trans*)-enoyl-CoA isomerase is required to convert the unsaturation at C3 to the *trans* form at the C2 position (Figure 2). Four genes encode this kind of isomerase in *P. putida* KT2440: *PP\_4030*, *PP\_3732*, *PP\_3726*, and *PP\_1845*. In the case of oleic acid, the  $\beta$ -oxidation intermediate 3-*cis*-dodecanoyl-CoA is isomerized to 2-*trans*-dodecanoyl-CoA to further continue with the cycle and synthesis of PHA monomers (Figure 2). By contrast, unsaturated fatty acids with a C=C bond extending from an even-numbered carbon atom require an NADPH-dependent 2,4-dienoyl-CoA reductase, encoded by *fadH* (*PP\_2008*), to be metabolized by  $\beta$ -oxidation.<sup>[120]</sup> For instance, *FadH* catalyzes the reduction of (2-*trans*,4-*cis*)-decadienoyl-CoA, a  $\beta$ -oxidation intermediate of linoleic acid, to 2-*trans*-decanoyl-CoA.<sup>[121]</sup> These two types of biochemical sequences are evidenced in the composition of the PHAs accumulated from oleic and linoleic acid. Polymers synthesized from oleic acid were composed of 3-hydroxy-5-*cis*-8-*cis*-tetradecadienoate and C6, C8, C10, and C12 saturated monomers, whereas the PHA produced on linoleic acid contained 3-hydroxy-6-*cis*-dodecenoate and 3-hydroxy-5-*cis*-8-*cis*-tetradecadienoate and C6, C8, and C10 saturated monomers.<sup>[121]</sup> Besides, *P. putida* KCTC2407 produced low amounts [2–3% (w/w)] of PHA containing unsaturated monomers when fed with either 10-undecenoic acid or 10-undecynoic acid as the sole carbon source. When grown with 10-undecenoic acid only, the copolyester consisted of 66% (mol/mol) 3-hydroxynonenoate, 24% (mol/mol) 3-hydroxyundecenoate, and 10% (mol/mol) 3-hydroxyheptenoate. Various molar mixtures of nonanoic and 10-undecenoic acid led to PHAs bearing C=C bonds accumulated at contents of  $\approx$ 37% (w/w). As observed with other additives, bioconversion of 10-undecenoic acid to polymers by *P. putida* was accelerated by the addition of nonanoic acid, which supports both cell growth and polymer production.

The material properties of polymers containing unsaturated monomers with double or triple bonds were significantly different from each other. PHA containing C=C bonds is transparent and highly sticky, whereas polymers bearing C≡C bonds were described as white and soft (Figure 5). Furthermore, the transparency and stickiness of C=C polymers were enhanced by increasing the proportion of unsaturated monomers. On the contrary, the properties of C≡C PHAs decreased as a function of the unsaturated monomer fraction. Due to their



Modified PHA-related substrates used for feeding	Novel mcl-PHA	Emerging properties	Production parameters range		
<b>Unsaturated fatty acids</b>					
 <p>C=C double bonds</p>		<p>Proposed as adhesives due to their high stickiness. Easily modified chemically to prepare new polymers.</p>	PHA concn. (g/L)	PHA % (w/w)	Total biomass (g/L)
			NR	15 – 35	NR
 <p>C≡C triple bonds</p>			NR	2 – 37	NR
<b>Halogenated alkanolic acids</b>					
 <p>X = Br, Cl or F Monohalogenated alkanolic acids</p>		<p>Properties of brominated and chlorinated PHAs unknown. Fluorinated polymers had Tms of 55-80°C, with fast crystallization rates from the melt and had high fusion enthalpies, and water-shedding properties.</p>	PHA concn. (g/L)	PHA % (w/w)	Total biomass (g/L)
			CI-PHA	0.13	5
 <p>Polyfluorinated alkanolic acids</p>			0.02 – 0.52	NR	NR
 <p>Mono and difluorinated phenoxyalkanoic acids</p> <p>F is either in the <i>ortho</i>, <i>meta</i> or <i>para</i> position of the aromatic ring in the monofluorinated substrates; F is in the <i>ortho</i> and <i>para</i> position in the difluorinated substrate</p>			0.003 – 0.006	6.5 – 9.7	0.05 – 0.09

**Figure 5.** Structure, properties, and production parameters of novel PHAs obtained by feeding of *P. putida* cultures with unsaturated fatty acids or halogenated alkanolic acids. Concn., concentration; NR, not reported.



physicochemical properties (especially, their sticky character), unsaturated PHAs were proposed as adhesive materials—and the ease of introducing chemical modifications makes them attractive to generate bioplastics with improved mechanical properties.<sup>[47]</sup>

PHAs bearing unsaturated monomers were also produced in *P. resinovorans* from soybean oil. These polymers were amorphous, indicating that the high degree of unsaturation in the side-chains inhibited crystallization. It has been suggested that saturated mcl-PHA crystallize with the participation of both the backbone and side-chains in a layer-like order.<sup>[122]</sup> The incorporation of *cis*-double C bonds into mcl-PHA results in distortions that disrupt this arrangement and allow for a large degree of conformational freedom. This feature results in decreased  $T_m$  and  $T_g$  values as side-chain unsaturation increases.<sup>[123]</sup>

### 8.1.6. Halogenated PHAs

The introduction of halogen atoms into polymers is a general strategy of chemical modification that has a deep impact on the physicochemical properties of materials.<sup>[124]</sup> Many halogenated polymers are extensively used nowadays for different applications.<sup>[125]</sup> Incorporation of bromine, for instance, leads to polymers with flame retardant properties that are used for developing inflammable materials. The chemical addition of chlorine atoms into the molecular structure of hydrocarbon polymers also confers useful properties. Polyvinylchloride, or PVC, has altogether different uses and applications than the nonchlorinated counterpart (polyethylene) of a similar structure. Yet, fluorine is the most widely used atom in polymer applications. Fluoropolymers present outstanding physicochemical properties, e.g., low coefficient of friction and low surface tension due to weak van der Waals forces, and chemical, thermal, and weathering resistance due to the stability of the C–F bonds. These remarkable features increase with the number of fluorine atoms present in the repeat unit. Along this line of reasoning, introducing halogens into PHA opens the door for the sustainable bioproduction of superior polymers with appealing material properties.

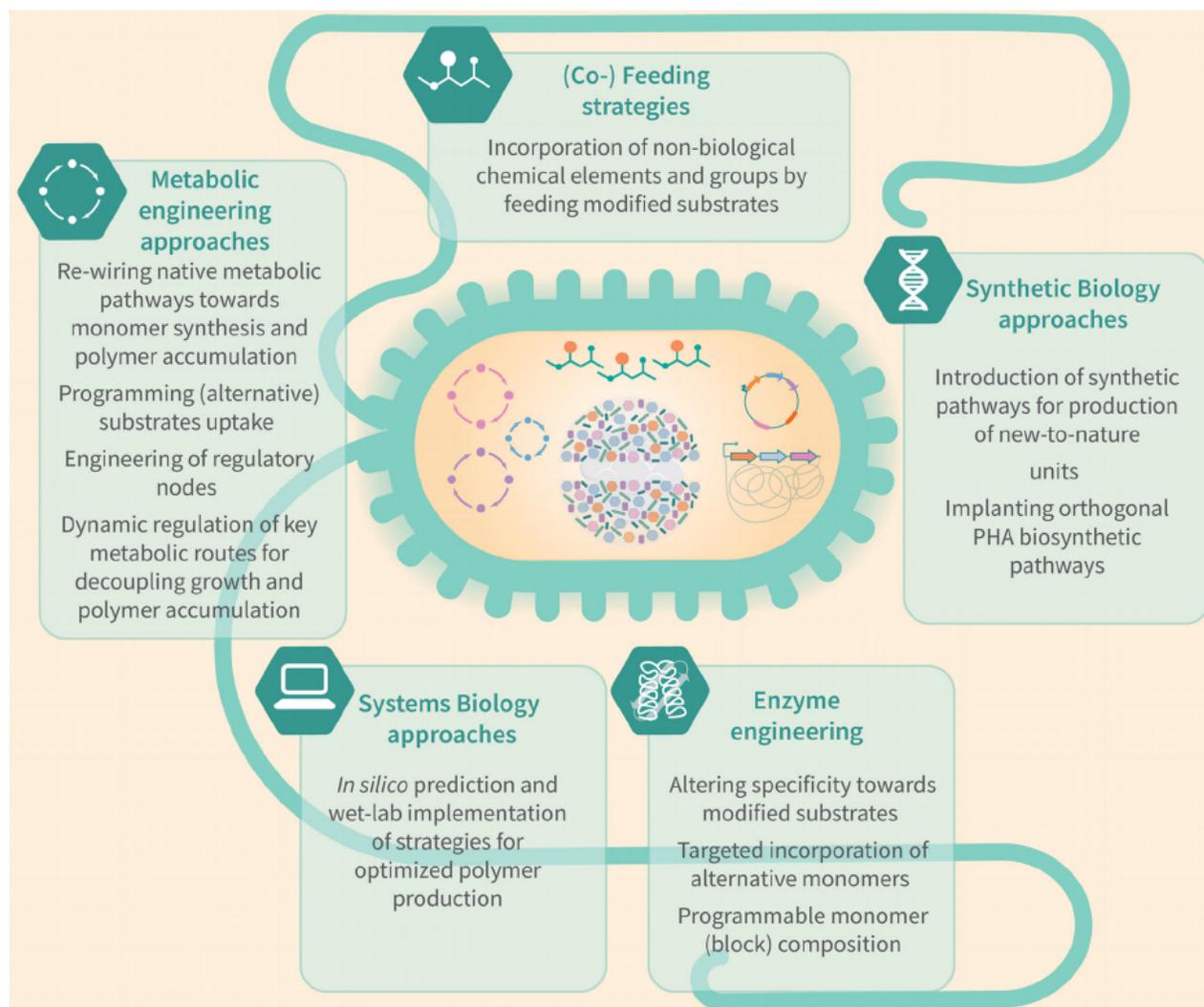
An early example of this sort has been the synthesis of PHAs containing monohalogenated side groups by *P. putida* Gp01 when incubated in the presence of  $\omega$ -monohalogenated alkanes (Figure 5). PHA copolymers containing brominated monomers were produced by the same species from mixtures of C6, C8 or C11  $\omega$ -bromoalkanoic acids and either nonanoic acid or octanoic acid. Remarkably, cells grown solely on a  $\omega$ -bromoalkanoic acid did not produce any PHA. On the contrary, PHAs containing both brominated monomers and alkyl monomers were obtained when using mixtures of brominated and nonhalogenated alkanic acids. All of the PHA copolymers therein had a single  $T_g$  and a single  $T_m$ , indicating that the monomers were randomly incorporated.<sup>[126]</sup> The same effect was observed when this strain was grown with octane and 1-chlorooctane, leading to the synthesis of a random copolyester of 3-hydroxyalkanoate and 3-hydroxy- $\omega$ -chloroalkanoate units in low amounts [0.13 g L<sup>-1</sup> and 5% (w/w)]. The polymer contained both C6 and C8 chlorinated units along with the nonhalogenated octanoate fraction.<sup>[127]</sup>

The addition of F to PHAs has been tested in *P. putida* Gp01 and *P. putida* KT2442 grown on fluorinated carbon sources

(Figure 5). The first evidence of the possibility of fluorinating PHA was obtained when *P. putida* Gp01 was cultivated on mixtures of 1-fluorononanoic and nonanoic acid. The copolyesters obtained contained up to 24% (mol/mol) of the fluorinated monomer, i.e., 3-hydroxy-9-fluorononanoate. Incorporation of monofluorinated repeat units into PHA polymers resulted in an increase in  $T_m$  from 47 to 61 °C and a slight decrease in  $T_g$  from –39 to –44 °C.<sup>[128]</sup> Further studies were performed to investigate if mcl-PHA-producing pseudomonads could metabolize substrates having a high degree of fluorination to form polyesters with 3-hydroxyalkanoate repeat units with such fluorinated side groups. Fluoroalkanoic acids with a different number of F atoms were synthesized chemically at a laboratory scale and tested as substrates for the production of fluorinated PHAs in *P. oleovorans* and *P. putida* KT2442. Examples of the substrates used to this end include 6,6,6-trifluorohexanoic, 6,6,7,7,8,8,8-heptafluorooctanoic, 6,6,7,7,8,8,9,9,9-nonafluorononanoic, and 6,6,7,7,8,8,9,9,10,10,11,11,11-tridecafluoroundecanoic acid. No PHA accumulation was observed when fluorinated substrates were used as the sole carbon source. Cells were first grown on sodium citrate, and the culture were then cofed with 1:1 molar mixtures of nonanoic acid and the different fluorinated alkanic acids in a second polymer producing stage. Notably, significant quantities of these fluoroalkanoates remained an insoluble phase in the culture medium. Cultivation of both *P. oleovorans* and *P. putida* on 1:1 mixtures of fluorinated acids with nonanoic acid resulted in substantial incorporation of fluorinated repeat units—except 6,6,7,7,8,8,9,9,10,10,11,11,11-tridecafluoroundecanoic acid that resulted in relatively high PHA yields but little to no incorporation of fluoromonomers. The highest level of incorporation of fluorinated side chains into PHA (17.3% mol/mol) was observed when *P. putida* was cultivated in the presence of nonanoic and 6,6,7,7,8,8,9,9,9-nonafluorononanoic acid as cosubstrates. The incorporation of fluoroalkanoate repeat units resulted in polymeric materials with  $T_m$ s of 55–80 °C that crystallize at faster rates compared to their nonfluorinated counterparts.<sup>[129]</sup>

*P. putida* 27N01 produced PHAs with fluorophenoxy side groups when grown on fluorophenoxyalkanoic acids. Fluorinated 11-phenoxyundecanoic acids containing one or two F atoms in the phenoxy group were tested as substrates for growth and polymer production. Monofluorinated substrates, e.g., 11-(2-fluorophenoxy)undecanoic, 11-(3-fluorophenoxy)undecanoic, and 11-(4-fluorophenoxy)undecanoic acid, stimulated the formation of almost-completely fluorinated PHAs, containing F in all repeating units—although the polymer content ranged from 3 to 6 mg L<sup>-1</sup> PHA [i.e., 6.5% to 9.7% (w/w)]. When cells were grown with 11-(2,4-difluorophenoxy)undecanoic acid, the homopolymer poly[3-hydroxy-5-(2,4-difluorophenoxy)pentanoate] was produced by the cells at 5 mg L<sup>-1</sup> [i.e., 8.5% (w/w)]. No PHA was produced when using tri- or tetra-fluorinated 11-phenoxyundecanoic acids. The number of substituent F atoms in the substrate was observed to decrease cell growth as compared to nonsubstituted phenoxyalkanoic acids. One way or the other, PHAs with a fluorinated side group exhibited  $T_m$ s at 52 °C (monofluorinated) and 102 °C (difluorinated), illustrating how F incorporation contributes to the improvement of the thermal properties. Besides, F dramatically affected physical properties, and difluorinated PHA was opaque,





**Figure 6.** Traditional and emerging strategies to manipulate PHA biosynthesis in *P. putida*-based cell factories. Novel approaches, rooted in Synthetic and Systems Biology, are currently guiding the efforts in the Metabolic Engineering of *Pseudomonas* species for bioproduction of tailored biopolymers. The incorporation of non-natural monomers, using a combination of these strategies, is of special interest toward blending nonbiological atoms into the biochemical pathways of the bacterial host.

cream-colored, possessed greater crystallinity and water-shedding properties.<sup>[130]</sup>

## 9. Fine-Tuning of PHA Synthesis: Metabolic Engineering Approaches in *P. putida* for the Production of Tailor-Made PHAs from Related and Nonrelated Substrates

### 9.1. Engineering of *P. putida* for PHA Production from Related Substrates

The first studies that explored the manipulation of PHA production and composition involved feeding strategies with substrates of different chemical nature, spanning different carbon chain length to achieve a synthesis of polymers with desired monomer composition (Figure 3). Synthetic biology brought novel tools for targeted genome engineering, opening up new scenarios full of

possibilities toward novel polymers. Increasing PHA titers and yields, fine-tuning of monomer composition and even rationally designing tailor-made PHAs with controllable thermal and mechanical properties for specific applications appeared as plausible outcomes (Figure 6).

Among the first engineering strategies explored for improving PHA synthesis from related substrates is the overproduction of enzymes that link  $\beta$ -oxidation and the PHA machinery to increase substrate channeling into polymer production. Over-expression of *phaJ* in *P. putida* KCTC1639 enhanced the biosynthesis of mcl-PHA from octanoate, increasing polymer content from 18% to 27% (w/w). These results support the previous observation that *phaJ* played an important role in PHA synthesis by converting the  $\beta$ -oxidation intermediate 2-*trans*-enoyl-CoA to HA-CoA.<sup>[68]</sup>

Another genetic manipulation that showed to have an impact on PHA synthesis is the deletion of *phaZ*, encoding PHA depolymerase. As synthesis and degradation of PHA are dynamic,



coupled biochemical processes that occur simultaneously for fulfilling cellular carbon and energy demands, suppression of PHA degradation was observed to increase PHA production. *P. putida* KT2440  $\Delta$ *phaZ* was shown to accumulate 86% (w/w) of mcl-PHA when cultured in a minimal medium containing sodium octanoate as the carbon source, whereas the wild-type strain produced 66% (w/w).<sup>[131]</sup> To study the role of expressing alternative PHA synthases in polymer composition, the complete native *phaC1ZC2* gene cluster was removed from *P. putida* KT2442 by functional replacement by *vgb* encoding the *Vitreoscilla* hemoglobin protein. This hemoglobin enhances oxygen uptake rates under low oxygen concentration, which resulted in enhanced growth under conditions with low oxygen availability. Genes encoding PHA synthases from different microorganisms, differing in their substrate specificity, were implanted in this engineered host and PHA production from dodecanoate was tested in the resulting strains. PHB was produced at very low amounts [i.e., 6.2% (w/w)] when expressing *phaC* from *C. necator* H16, which incorporates C4 monomers. Poly(3HB-co-3HHx) was accumulated up to 6.8% (w/w) when *phaC* from *Aeromonas caviae*, which favors C4 and C6 monomers, was expressed instead. *phaC* from *P. stutzeri* 1317, which has low substrate specificity, led to the formation of PHA with a wide range of monomers from C4 to C12 at 31.8% (w/w). Furthermore, coexpression of *phaC* from *P. stutzeri* and *phbAB* from *C. necator* was observed to increase the fraction of C4 monomers.<sup>[132]</sup>

One of the most extensively used metabolic engineering approaches for boosting mcl-PHAs production from related substrates in *P. putida* strains is the manipulation of genes encoding  $\beta$ -oxidation enzymes to create  $\beta$ -oxidation-weakened mutants. Such manipulations not only allowed for increased PHA accumulation and introduction of monomers with longer carbon chains, but they also contributed to the understanding of  $\beta$ -oxidation in *P. putida* KT2442 and its involvement in PHA synthesis. One of the first approaches to weaken the  $\beta$ -oxidation pathway was deleting FadB variants, the enzyme with multiple activities that converts the enoyl intermediate to 3-ketoacyl-CoA in two steps (Figure 2). *FabA*, catalyzing the transformation of 3-ketoacyl-CoA to acyl-CoA  $n-2$ , has been likewise manipulated. Three mutant strains of *P. putida* KT2442 were constructed to study the impact of impaired  $\beta$ -oxidation on PHA production and to understand the role of the two sets of *fadBA* gene clusters, i.e., i)  $\Delta$ *fadBxAx*, ii)  $\Delta$ *fadBA*, and iii)  $\Delta$ *fadBxAx*  $\Delta$ *fadBA*. When grown in dodecanoate, cell growth and PHA accumulation in the wild-type strain and its  $\Delta$ *fadBxAx* derivative was almost the same [ $\approx$ 50% (w/w)], indicating that this operon is not relevant for fatty acid-dependent growth. On the contrary, the *fadBA* deletion strain grew slower and reached lower biomass concentrations than the other strains, but it accumulated higher amounts of the polymer [69% (w/w)] with increased 3HDD content (from 7.5% mol/mol in the wild-type to 35.7% mol/mol in the mutant). FadBA thus plays a key role in  $\beta$ -oxidation and eliminating these orthologues allows incorporating monomers with long carbon chains. PHA produced by the  $\Delta$ *fadBA* strain still contained monomers shorter than C12, indicating that  $\beta$ -oxidation was weakened but not suppressed. The  $\Delta$ *fadBxAx*  $\Delta$ *fadBA* mutant had the slowest growth among the strains tested, and showed a reduced PHA producing capacity [accumulating only 19.8% (w/w)]. Overall, these results evidenced the existence of overlap-

ping functions of redundant  $\beta$ -oxidation functions and indicated that FadBxAx became more relevant in the absence of FadBA. Furthermore, weakened  $\beta$ -oxidation re-directed carbon fluxes to PHA synthesis while limiting fluxes toward cell growth—thus augmenting the polymer content on biomass.  $\beta$ -oxidation-weakened strains produced a PHA with a high 3HDD content, a polymer with higher crystallinity and tensile strength than the PHA accumulated by the wild-type strain.<sup>[63a]</sup>

Further manipulations were introduced in the *fadBxAx fadBA* context. FadB is predicted to also act as a 3-hydroxyacyl-CoA dehydrogenase, however, this transformation can also be performed by the product of *PP\_2047*, involved in  $\beta$ -oxidation of long-chain fatty acids. When this gene was partially or completely deleted, the PHA accumulated from C8, C10, and C12 fatty acids contained only two different monomers, dominated by monomers of the same length as the substrate and another unit that was two carbon atoms shorter. The copolymer containing 44% (mol/mol) C10 units and 56% (mol/mol) C12 units showed a  $T_m$  of 75 °C—higher than the values reported for PHAs produced by the wild-type strain, with a  $T_m < 50$  °C.

Further deletions were introduced in this strain to investigate the possibility of further reduce the activity of the  $\beta$ -oxidation and to produce a PHA homopolymer containing long-chain length monomers. In particular, *PP\_2048* (encoding a putative acyl-CoA dehydrogenase) and *phaG* were deleted to obtain a strongly suppressed  $\beta$ -oxidation and to completely avoid FAS-derived monomers, respectively. The resulting strain synthesized poly(3-hydroxydecanoate) ( $T_m = 72$  °C,  $T_g = 37.21$  °C) from decanoic acid, and poly(3HD-co-3HDD) with an 84.5% (mol/mol) fraction of 3HDD ( $T_m = 78$  °C,  $T_g = 32.49$  °C) when grown in dodecanoic acid. These novel PHA were stable at 250 °C and all  $T_g$ s were 7–11 °C higher than conventional mcl-PHA produced by the wild-type strain, which are mostly amorphous.<sup>[133]</sup> Polymers obtained through weakened  $\beta$ -oxidation mutants showed improvements in  $\sigma^t$ ,  $e^b$ , and  $E$  values. A higher percentage of long-chain length monomers (e.g., C12 and C14) in mcl-PHA copolymers significantly improved thermal and mechanical properties.<sup>[133,134]</sup> These PHAs showed higher crystallinity, but they behaved as thermoplastic elastomers with good  $\sigma^t$  and desirable  $e^b$ . They also display an increased  $E$  value that has been demonstrated to facilitate processing and moulding.<sup>[63a,133]</sup>

Besides the incorporation of longer carbon chain monomers into PHAs, weakening  $\beta$ -oxidation was used for the synthesis of novel PHAs. Poly(3-hydroxyphenylalkanoates), containing monomers with high carbon chain lengths, were produced when the gene that encodes the 3-ketoacyl-CoA thiolase (*fadA*) of *P. putida* U was disrupted. If the resulting strain was cultured in the presence of the  $\omega$ -phenyl derivatives of fatty acids 6-phenylhexanoic, 7-phenylheptanoic, or 8-phenyloctanoic acid as the carbon source, amorphous poly(3-hydroxyphenylalkanoates) with distinct material properties were produced. When 6-phenylhexanoic acid was added to the cultures, the homopolymer poly(3-hydroxy-6-phenylhexanoate) ( $T_g = -1.3$  °C) was accumulated, whereas the feeding with 8-phenyloctanoic and 7-phenylheptanoic acid led to the formation of random copolymers presenting the same carbon chain length as the carbon source ( $n$ ) and monomers with  $n-2$  carbons units. As observed before, the  $T_g$ s of these polymers (−14.8 and −11.2 °C, respectively) were relatively low, due to the increased flexibility caused by



introducing alkyl side chains longer than  $-(\text{CH}_2)_3$ . Because of their physicochemical properties, these copolymers were proposed for the design of new controlled-delivery systems or the preparation of composites for biomedical applications.<sup>[109]</sup>

*P. putida* KT2442  $\Delta$ *fadBxAx*  $\Delta$ *fadBA* was further engineered by knocking-out *phaG* and *phaC*, followed by the introduction of *phaC* from *C. necator* and *orfZ* of *Clostridium kluyveri* (encoding a 4-hydroxybutyrate-CoA transferase to enable incorporation of scl-monomers). The resulting strain was able to convert fatty acids into C4 scl-PHA due to the specificity of PhaC from *C. necator* toward such monomers. When grown on butyrate, this strain produced PHB only, while poly(4-hydroxybutyrate) was the only polymer produced when the engineered strain was grown on  $\gamma$ -butyrolactone. An scl-PHA block copolymer, consisting of PHB and poly(4-hydroxybutyrate) as the individual blocks, was produced by cofeeding with both C4 substrates. Physicochemical properties could be manipulated by altering the ratio of the two blocks via substrates composition.<sup>[135]</sup>

*P. putida* KT2442  $\Delta$ *fadBA* was further engineered for scl-monomers incorporation. To this end, genes encoding endogenous PHA synthases and depolymerases were deleted and the PHB biosynthesis genes from *Aeromonas caviae* (*phaPCJ<sub>Ac</sub>*) were plugged-in. When fed with different mixtures of butyrate and heptanoate (at different C4/C7 ratios) the resulting strain produced random copolymers consisting of C4, C5, and C7 units in a proportion according to the C4/C7 ratio. Using a two-step cultivation approach, this strain was able to produce a block copolymer consisting of 70% (mol/mol) PHB as one block and around 30% (mol/mol) of a random mcl-copolymer of 3HV and 3HHp as another block. Cells were first grown on butyrate as a PHB precursor. After exhaustion of butyrate, heptanoate was fed to the culture to produce the mcl-component.<sup>[136]</sup> The block and random copolymers showed completely different thermal behaviors due to their different chain structure; the first one having two  $T_g$ s ( $-23.6$  and  $3.5$  °C) and a  $T_m = 170.6$  °C, whereas the second had a single  $T_g$  ( $-7.3$  °C) due to the random assembly of its components in the chain segment.<sup>[136]</sup> Furthermore, when this strain was fed with variable ratios of butyrate and hexanoate (C4/C6) or hexanoate and decanoate (C6/C10), a plethora of random copolymers containing C4 and mcl-units with variable composition was produced. The sequential addition of structurally-related C6 and C12 substrates fostered the synthesis of the diblock copolymer poly(3HHx-block-poly(3HD-co-3HDD)) consisting of 49% and 51% (mol/mol) of each block [the second one with 5.86% (mol/mol) C10 and 35.25% (mol/mol) C12]. This complex PHA had a  $T_g = 43.08$  °C and two  $T_m$ s of 33.45 and 66.08 °C. All these examples illustrate how  $\beta$ -oxidation-weakened *P. putida* has the capability of producing a variety of block and random polymers, combining the amorphous nature of mcl-PHAs to the crystallinity of scl-PHAs.<sup>[137]</sup> Moreover, this strategy was also exploited for the production of modified PHAs containing monomers with longer aliphatic chains with a number of chemical decorations.<sup>[109,118]</sup>

Novel PHAs containing scl-monomers encompass the incorporation of C3 (lactyl) units. *P. putida* KT2440 was modified for this purpose by plugging-in i) a codon-optimized *pct* from *Clostridium propionicum*, encoding an engineered propionate-CoA transferase to synthesize (R)-lactoyl-CoA and ii) a modified PHA synthase from *Pseudomonas* sp. MBEL 6–19 (PhaC1<sub>P56-19</sub>),

previously engineered to display a broader substrate specificity and able to recognize (R)-lactoyl-CoA as substrate. Both genes were cloned in a plasmid and transformed in strain KT2440 and its  $\Delta$ *phaC* derivative. When grown on octanoate or lactate as the sole carbon source, these engineered strains accumulated the novel quaterpolymer poly(3HB-co-lactate-co-3HHx-co-3HO). Octanoate performed better than lactate for cell growth and polymer production (42% (w/w) from octanoate and  $\approx$ 5% (w/w) from lactate), growth on C3 supplied more precursors for scl-PHA. This implies that low amounts of lactyl-CoA are produced by *P. putida* during growth on octanoate. In addition, the fraction of C3 and C4 units was increased in the  $\Delta$ *phaC* mutant complemented with *phaC1<sub>P56-19</sub>* compared to the strain retaining the native *phaC* genes, probably due to competition between the two polymerases in the wild-type strain.<sup>[138]</sup>

## 9.2. Engineering of *P. putida* for PHA Production from Nonrelated Substrates

Most of the previous strategies for improved natural and novel PHA synthesis required the external addition of substrates that are structurally related to the monomers of interest, mostly in the form of modified fatty acids. Such substrates are expensive and several of them had to be chemically synthesized, which would be impractical at a scale larger than the laboratory. By contrast, nonrelated substrates are usually cost-effective and readily available. On this line of reasoning, *P. putida* has been also engineered for PHA production from nonrelated substrates. One of the first identified targets for genetic manipulation was found by transposon mutagenesis of *P. putida* KT2442. One of the mutants with increased mcl-PHA accumulation had an insertion in *aceA*, encoding isocitrate lyase. Inactivation of the glyoxylate shunt and reduction of the isocitrate dehydrogenase activity in this strain resulted in a 1.7-fold increase in mcl-PHA accumulation from gluconate as compared to the wild-type strain. Since PHA synthesis competes with the TCA cycle for acetyl-CoA as a precursor, either the level of citrate synthase activity or oxaloacetate availability should be lowered to prevent acetyl-CoA from entering the TCA cycle (Figure 2). As such, the observed phenotype was related to a reduction of the intracellular oxaloacetate concentration arising from either i) cutting off the supply of malate, its direct precursor, thereby blocking the glyoxylate pathway or ii) preventing isocitrate processing by limited isocitrate dehydrogenase activity.<sup>[95a]</sup>

The synthesis of new types of scl-PHAs from nonrelated substrates has also been achieved in *P. putida* KT2440. scl-PHA was produced using levulinic acid, a C5 compound derived from biomass-based sugars with high industrial value due to its relatively limited toxicity and low price without competing with food resources. This strain was the host of choice because of its ability to grow efficiently on levulinic acid as a sole carbon source. The catabolism of this compound is mediated by the products of a seven-gene *lva* operon (*lvaABCDEFGF*, *PP\_2791-PP\_2797*) that generate intermediates entering  $\beta$ -oxidation and furnishing monomers for scl-PHA. The substrate is first converted into 4-hydroxyvaleryl-CoA by LvaED and then transformed into 3-hydroxyvaleryl-CoA through the action of LvaABC. 3-Hydroxyvaleryl-CoA can be fully catabolized by  $\beta$ -oxidation to



propionyl-CoA and acetyl-CoA, entering the TCA cycle for complete oxidation or channeled to PHA synthesis via PhaJ.<sup>[139]</sup>

Taking advantage of this endogenous pathway, *P. putida* can be engineered to produce scl-PHA from monomers of levulinic acid degradation. First, the native PhaC1ZC2 machinery was eliminated in reduced-genome *P. putida* EM42 to block mcl-PHA production and prevent scl-PHA degradation. Second, different synthetic scl-PHA pathways were implanted in this chassis. PhaEC from *Thiocapsa pfennigii*, a class I PHA synthase with broad substrate specificity, and three class III PHA synthases from *C. necator* H16, *Chromobacterium violaceum*, and *Paracoccus denitrificans* (with a relatively high preference for 3- or 4-hydroxyvaleryl-CoA) were plugged-in in the  $\Delta$ *phaC1ZC2* strain. Additionally, these synthases were further coexpressed with *phaBA*, encoding acetyl-CoA acetyltransferase or thiolase (PhaA), and acetyl-CoA reductase (PhaB) from *C. necator* to provide 3-hydroxybutyryl-CoA as an extra monomer for scl-PHA synthesis. Genes encoding enzymes of these synthetic pathways were cloned under the regulation of the *lvaA* promoter that can be induced by the substrate. The engineered strains were cofed with glucose and levulinic acid to support growth and PHA production, and four different types of scl-PHA copolymers were produced. 3HB, 3HV, and/or 4-hydroxyvalerate were the units, incorporated at different fractions depending on the expression of *phaAB* and the PHA synthase of choice.<sup>[140]</sup>

*P. putida* KT2442 has also been engineered for the production of 4-hydroxybutyrate, the monomer of poly(4-hydroxybutyrate). This homopolymer has unique properties that make it suitable for biomedical applications (e.g., as absorbable material for implants and sutures). A very distinct characteristic of poly(4-hydroxybutyrate) is that it can be stretched to ten times its original length without breaking and is the only PHA-based material with FDA clearance for clinical usage.<sup>[141]</sup> Overproduction of the native enzymes 1,3-propanediol dehydrogenase, encoded by *dhaT* (*PP\_2803*), and aldehyde dehydrogenase, encoded by *aldD* (*PP\_0545*), led to the production of 4-hydroxybutyrate from 1,4-butanediol. DhaT catalyzes the conversion of 1,3-propanediol into 3-hydroxypropionaldehyde, but it was also observed to convert 1,4-butanediol to 4-hydroxybutyraldehyde, that can be further transformed to C4 units by AldD. As DhaT catalyzes a bidirectional reaction, overexpression of *aldD* is also important to pull the reaction toward the 4-hydroxybutyrate formation. A strain overexpressing *dhaT* and *aldD* in a plasmid grew to 1.8 g L<sup>-1</sup> biomass and produced 4.6 g L<sup>-1</sup> of 4-hydroxybutyrate in the supernatant when using 1,4-butanediol as carbon additive in LB medium. *P. putida* has not been further engineered for the production of polymers containing 4-hydroxybutyrate, mainly produced using recombinant *E. coli* strains.<sup>[142]</sup> Metabolic engineering approaches have been used to optimize PHA synthesis from lignin derivatives. Overexpression of *phaG* and *alkK* in a plasmid, cloned under the control of a strong inducible promoter, led to an increase in PHA production from vanillic acid in *P. putida* A514.<sup>[102]</sup> Also, *phaZ* was deleted in a  $\beta$ -oxidation-reduced chassis, while *phaG*, *alkK*, *phaC1*, and *phaC2* were introduced in the chromosome and overexpressed to increase carbon flux into mcl-PHA biosynthesis via FAS. Strains were evaluated on *p*-coumaric acid and in complex lignin streams (containing aromatic compounds, acetate, and sugars) and had a 1.53- and 2-fold increase in mcl-PHA titer and a 1.2- and a 2-fold increase

in PHA content from *p*-coumaric acid and lignin, respectively, as compared to the wild-type strain.<sup>[95b]</sup>

### 9.3. PHA Synthases of *Pseudomonas* as SynBio Targets

PHA synthases are classified into four groups based on primary structure and substrate specificity: types I, III, and IV can accommodate C3 to C5 monomers (resulting in scl-PHA), whereas type II synthases (among them, variants present in *Pseudomonas*) can accept C6 to C14 monomers, with a preference for C10 and C8 units (thus giving rise to mcl-PHA).<sup>[20a,143]</sup> mcl-PHA-producing *Pseudomonas* species are usually equipped with two polymerases, PhaC1 and PhaC2, and the presence of just one polymerase is generally sufficient for polymer biosynthesis. Although both enzymes display a comparable substrate specificity, studies with *P. putida* GPp104  $\Delta$ *phaC1C2* showed that the C6 content of PHA increased by overexpressing *phaC1*, whereas expression of *phaC2* favored the incorporation of C8 units.<sup>[37]</sup> Unexpectedly, increased synthase production did not translate into higher levels of product and even resulted in polymers with decreased molecular weight. As the substrate/enzyme ratio determines the molecular weight of PHA, the availability of polymer precursors seems to be limiting when the synthase is overproduced.<sup>[20a,138,144]</sup>

Furthermore, PHA synthases have been modified by evolution and semirandom mutagenesis. To this end, synthetic PHA synthases, containing sequences from different individual enzymes, were constructed to obtain variants with improved catalytic activity. These strategies aim at producing high PHA titers and/or to alter the substrate specificity, enabling biosynthesis of PHAs with broader monomeric composition.<sup>[102,145]</sup> A few natural PHA synthase variants with broad substrate specificity have been found in *Pseudomonas* species. PhaC1<sub>P56-19</sub> from *Pseudomonas* sp. MBEL 6–19 (isolated from activated sludge), PhaC2<sub>P81317</sub> from *P. stutzeri* 1317, and PhaC<sub>61-3</sub> from *Pseudomonas* sp. 61–3 (isolated from the soil), present broad substrate specificities toward C4–C12 3-hydroxyacyl-CoAs and enable the production of copolymers with varying amounts of scl- and mcl-monomers.<sup>[146]</sup> Variants with even larger substrate specificity will be of great importance for the production of tailored PHAs from modified substrates.

### 9.4. Optimization of PHA Production by Systems Biology Strategies

The increasing interest in combining systems biology and SynBio triggered the adoption of design–build–test–learn (DBTL) cycles toward bioprocess optimization. These iterative designs help the automation of essentially any given objective, starting with experimental design and in silico data simulation (design), rational strain modification and deployment of DNA constructs (build), high-throughput phenotyping (test), and data analysis (learn).<sup>[147]</sup> Several DBTL rounds can be performed to achieve the proposed objective, as elegantly illustrated for the production of dodecanol in engineered *E. coli*.<sup>[148]</sup>

PHA production by *Pseudomonas* species has been (and continues to be) an attractive target to deploy this strategy. In silico-driven systems, metabolic engineering has been applied in the last years to predict gene targets of deletion or overexpression toward biopolymer production from cheap nonrelated



substrates. The genome of *P. putida* KT2440 is completely sequenced and available since 2002<sup>[14e,19b]</sup>—encoding all the genes needed for mcl-PHA biosynthesis—and central metabolic pathways are well known, making it possible to use models to re-engineer metabolic networks toward improved PHA production from inexpensive substrates. Following this premise, the use of GSMMs for rational metabolic engineering of *P. putida* has become a common practice in the scientific community. As indicated above, GSMMs are organism-specific knowledge databases including detailed information about biochemical, genetic, metabolic, physiological, and multiomic data.<sup>[149]</sup> The metabolism of *P. putida* KT2440 has been characterized, and eight GSMMs are currently available (i.e., iJN746,<sup>[14a]</sup> iJP850,<sup>[14b]</sup> PpuMBEL1071,<sup>[14c]</sup> iJP962,<sup>[14d]</sup> iEB1050,<sup>[14e]</sup> PpuQY1140,<sup>[14f]</sup> iJN1411, and iJN1462<sup>[14g]</sup>). These GSMMs have been used to study important metabolic features of *P. putida*, e.g., defining and cataloging conditionally essential genes<sup>[150]</sup> and shedding light on the complex carbon catabolite regulatory systems.<sup>[151]</sup> Despite the progress, *P. putida* GSMMs have not been exploited to the extent seen for other bacterial species—e.g., *E. coli*, where GSMMs have already been tested for a wide variety of applications.<sup>[152]</sup> GSMMs can simulate PHA biosynthesis by strain KT2440 from several substrates—yet a missing piece of information in such interpretations is the complex PHA regulation network, which cannot be captured by most of the currently available models. Recent GSMMs versions, however, started to incorporate more accurate networks and can simulate (to some extent) biosynthesis of PHA with different monomer patterns.

A remarkable example of this approach has been the in silico-guided construction of *P. putida* strains optimized for PHA production from sugars, where in silico predictions were successfully validated in wet-lab experiments.<sup>[153]</sup> Unforeseen targets for metabolic engineering were discovered by using this approach. Glucose dehydrogenase, encoded by *gcd* (*PP\_1444*), which catalyzes glucose oxidation to gluconate in the periplasm, was predicted as a high-priority target to prevent undesired by-products formation and excretion that would misroute sugars away from PHA biosynthesis. The resulting  $\Delta gcd$  mutant indeed had increased PHA biosynthesis performance in shaken-flask and bioreactor fermentations—PHA content increased by 60% and 100%, respectively, as compared to the parental strain grown under the same conditions.<sup>[153a]</sup> These promising results pave the way for further application of GSMMs toward the valorization of complex substrates (e.g., PET or lignin) into biopolymers. Furthermore, GSMMs have been also exploited to model PHA production in microbial communities where *Pseudomonas* species are present<sup>[154]</sup>—conditions where the degradation of complex substrates would probably be easier than in individual species cultures.

In addition to GSMMs, a *P. putida* kinetic model has been used for predicting metabolic fluxes during PHA production.<sup>[189a]</sup> More recently, several kinetic models were also obtained using iJN1411 as the basis,<sup>[155]</sup> encompassing refined annotations and a more accurate definition of enzyme parameters that would surely have an impact on upgrading the quality of in silico predictions. Although this practice is becoming more and more commonplace, the systematic use of GSMMs to guide engineering strategies is still in its infancy, offering countless opportunities not only to improve PHA production—but also to explore and learn more

about general principles of metabolic regulation in *P. putida*. Besides the core biochemical properties of this bacterium that can be extensively manipulated, other aspects are equally relevant for biopolymer production—such as the bioprocess strategies discussed below.

### 9.5. High-Cell-Density Cultivation of *P. putida* for PHA Production

Improvement of biopolymer synthesis in *P. putida* not only requires strain engineering approaches, but also the development of cost-effective bioreactor cultivation processes to achieve high biomass concentrations and commercial-scale production of PHAs. High-cell-density cultivation approaches were shown to be effective for achieving high volumetric productivity of PHAs. A particularly successful approach consisted of applying phosphorus limitation in the fed-batch culture of *P. putida* KT2442 by reducing the initial concentration of phosphates. A final cell dry weight of 141 g L<sup>-1</sup> and a PHA content of 51.4% (w/w) were obtained after 38 h, resulting in high volumetric productivity of 1.91 g PHA L<sup>-1</sup> h<sup>-1</sup>.<sup>[87b]</sup> Another strategy consisted of a fed-batch bioprocess with *P. putida* KT2440 that did not require supplying oxygen-enriched air, using glucose as the sole carbon and energy source. A final cell dry weight of 102 g L<sup>-1</sup> was achieved in 33 h (with biomass productivity of 3.1 g L<sup>-1</sup> h<sup>-1</sup>) by following this protocol. Furthermore, when nonanoic acid was cofed to glucose-grown cells, 32% (w/w) PHA was accumulated in 11 h (i.e., volumetric productivity of 2.85 g PHA L<sup>-1</sup> h<sup>-1</sup>)—resulting in a total of 0.56 kg of PHA in 18 L of culture broth, with a yield of 0.56 g PHA/g nonanoic acid.<sup>[156]</sup> More recently, a reactive pulse-feed strategy, based on real-time measurements of CO<sub>2</sub> evolution or dissolved oxygen concentration as feedback variables, was used to control an oxygen-limited fed-batch process with *P. putida* LS46. Total biomass of 28.8 g L<sup>-1</sup> and a PHA content of 61% (w/w) were achieved after 27 h using octanoic acid as the carbon source in a bioreactor operated under atmospheric conditions—resulting in final volumetric productivity of 0.66 g PHA L<sup>-1</sup> h<sup>-1</sup>.<sup>[157]</sup> As such, high-cell-density cultivation processes, combined with the synthetic biology, metabolic engineering, and systems biology approaches—as summarized in the previous sections of this review—can help to overcome the long-standing problem of low PHA titers. This aspect is particularly important for producing novel biopolymers (which are typically accumulated in limited amounts) to achieve an economically competitive industrial scale.

## 10. Outlook and Perspectives

The vast amount of primary literature describing PHA biosynthesis in *Pseudomonas* species bears witness to the metabolic versatility of this host, which, over time, enabled the use of a wide variety of substrates to drive (and alter) polymer production. In this sense, the adoption of recalcitrant compounds (the degradation of which involves harsh biochemical transformations) as carbon sources, and feeding substrates modified with non-natural chemical groups are the most common examples found in the literature. Yet, many challenges still need to be solved to



achieve efficient biopolymer production. One of the main drawbacks to overcome is the very low titers of novel PHAs produced using feeding strategies—due, in part, to the toxicity of substrates or their slow and inefficient conversion to PHA precursors. Furthermore, the incorporation of modified monomers is still not programmable and depends on intricate regulatory signals in the host (some of which still elude full understanding, let alone engineering). This hurdle is a key target for smart metabolic engineering and SynBio approaches in the near future. In connection with this issue, all the chemical groups introduced into polymers produced by *Pseudomonas* are inevitably displayed in the side chain of the polymer. It would be critical to engineering the addition of chemical groups in the *backbone* of PHAs to bestow emerging material properties. Experimental evidence supporting this notion is a recent study demonstrating that F atoms can be linked to the backbone of PHB in engineered *E. coli* strains externally fed with 2-fluoromalonnate.<sup>[158]</sup> Again, a fully reprogrammed metabolism in *Pseudomonas* will be critical to achieving this purpose—including the deployment of synthetic metabolic modules that can be externally controlled at the user's will to insulate PHA precursors from the rest of the metabolism, thereby avoiding substrates misrouting. The recently demonstrated implantation of neometabolism for in vivo biofluorination in *P. putida*<sup>[159]</sup> will trigger further developments in this direction.

Besides metabolic manipulations in the host and altering the feeding composition, other challenges should be likewise tackled in the near future to position PHAs as a solid alternative to traditional plastics. A long-standing issue is our inability to precisely control monomer enrichment in PHAs. Owing to the inevitable cross-talk between central catabolic routes and PHA metabolism, coupled to the specificity of PHA synthases, multiple monomers end up being incorporated into PHA randomly. Biomedical applications demand a defined, tightly controlled monomeric composition, and approval of PHA uses by regulatory organizations (e.g., the FDA) requires a consistent molecular weight of the corresponding materials. Again, advanced SynBio approaches could help to fix this caveat in PHA production by *Pseudomonas*—especially when it comes to the biosynthesis of copolymers, a signature trait of these species—by designing PHA synthases that could fulfill these demands. Yet another level of regulation is offered by manipulating GAPs (i.e., not only PHA biosynthesis but also structural proteins that shape polymer inclusions). The development of synthetic phasins and protein tags are increasingly gaining popularity as a strategy to control PHA formation and stability in vivo—an approach that will become essential for producing novel biopolymers, which will probably interact with other macromolecular components inside the cell cytoplasm in a difficult to predict fashion. One way or the other, the field of polymer production by *Pseudomonas* is far from spent: on the contrary, the emergence of novel approaches to control PHA biosynthesis and composition is burgeoning—and will become instrumental in our quest for a true circular bioeconomy to reduce the environmental footprint of modern society.<sup>[160]</sup>

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## Conflict of Interest

The authors declare no conflict of interest.

## Data Availability Statement

Not applicable.

## Author Contributions

M.P.M. contributed to conceptualization-equal, writing-original draft-equal; M.T.M. to writing-original draft-equal; M.A.P. to conceptualization-equal, writing-original draft-equal; P.I.N. to conceptualization-equal, funding acquisition-lead, project administration-lead, supervision-lead, writing-review, and editing-lead.

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