CHAPTER THREE

Polyhydroxyalkanoates: Much More than Biodegradable Plastics

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Abstract

Bacterial polyhydroxyalkanoates (PHAs) are isotactic polymers that play a critical role in central metabolism, as they act as dynamic reservoirs of carbon and reducing equivalents. These polymers have a number of technical applications since they exhibit thermoplastic and elastomeric properties, making them attractive as a replacement of oil-derived materials. PHAs are accumulated under conditions of nutritional imbalance (usually an excess of carbon source with respect to a limiting nutrient, such as nitrogen or phosphorus). The cycle of PHA synthesis and degradation has been recognized as an important physiological feature when these biochemical pathways were originally described, yet its role in bacterial processes as diverse as global regulation and cell survival is just starting to be appreciated in full. In the present revision, the complex regulation of PHA synthesis and degradation at the transcriptional, translational, and metabolic levels are explored by analyzing examples in natural producer bacteria, such as *Pseudomonas* species, as well as in recombinant *Escherichia coli* strains. The ecological role of PHAs, together with the interrelations with other polymers and extracellular substances, is also discussed, along with their importance in cell survival, resistance to several types of environmental stress, and planktonic-*versus*-biofilm lifestyle. Finally, bioremediation and plant growth promotion are presented as examples of environmental applications in which PHA accumulation has successfully been exploited.

1. INTRODUCTION

Many bacterial species synthesize polyhydroxyalkanoates (PHAs) as carbon and energy storage compounds under growth conditions characterized by an abundance of carbon sources with respect to other nutrients, such as nitrogen or phosphorus. The physicochemical properties of these polymers vary depending on the size of the monomer. The most common PHA is poly(3-hydroxybutyrate) (PHB), but bacteria can accumulate PHAs with monomers of lengths between 3 and 20 C atoms. Polymers composed by C3–C5 monomers are called short chain length PHAs (sclPHAs), whereas medium chain length PHAs (mclPHAs) contain C6–C14 monomers. Long chain length PHAs have monomers > C14 (Madison & Huisman, 1999). These polymers continue to attract increasing industrial interest as renewable, biodegradable, biocompatible, and extremely versatile thermoplastic and elastomeric materials. Several reviews have analyzed the complex function landscape of these multipurpose materials (Keshavarz & Roy, 2010; Nigmatullin, Thomas, Lukasiewicz, Puthussery, & Roy, in press). The biochemistry and molecular biology of PHA synthesis and degradation in several bacterial species have also been elucidated (Madison & Huisman, 1999; Pötter & Steinbüchel, 2005). PHAs are accumulated intracellularly as complex inclusion bodies

or granules. The granules are surrounded by attached proteins that include, among others, the PHA synthase, depolymerizing enzymes, regulatory proteins, and structural proteins called phasins (Grage et al., 2009). This multicomponent structure has recently been designed as carbonosome, a name that reflects its multifunctionality (Jendrossek & Pfeiffer, 2014). These polymers can be considered suitable alternatives to fossil fuel-derived plastics, but some problems associated with their industrial production must be contemplated, such as the high production cost of PHAs and the lack of sustainability of the fermentation process. This subject has been addressed by different strategies that rely on the functionalization of the polymers to increase their value (Andreeβen, Taylor, & Steinbüchel, 2014; Dinjaski & Prieto, 2015; Li & Loh, 2015; Tortajada, Ferreira da Silva, & Prieto, 2013), the optimization of producing strains (Brigham, Zhila, Shishatskaya, Volova, & Sinskey, 2012; Leong, Show, Ooi, Ling, & Lan, 2014; Wang, Yin, & Chen, 2014), the use of industrial residues as substrates, or the development of energy-saving processes (Gómez et al., 2012; Nikodinovic-Runic et al., 2013).

In this review, we will address the physiological aspects of PHAs that have not yet received special attention. The role of the polymer in the multiple strategies developed by bacteria to increase survival and stress tolerance will be described, including the acquisition of *pha* genes by horizontal gene transfer, together with the effect that this multicomponent activity has in bioremediation and growth promotion of different bacteria. The emerging picture of global regulation of the expression of *pha* genes and the integration of PHAs in bacterial metabolism will also be presented, revealing the multiple aspects of this key component in bacterial carbon homeostasis.

2. STRESS RESISTANCE AND SURVIVAL

In the late 1970s, a seminal work by Matin, Veldhuis, Stegeman, and Veenhuis (1979) clearly showed the positive role of PHB in the survival of bacteria. A *Spirillum* sp. strain, a PHB producer, was isolated from a freshwater pond with limited nutrients and was grown in the laboratory in chemostats at different dilution rates (*D*) followed by starvation experiments. The control experiments selected by the authors consisted of similar cultures of a *Pseudomonas* sp. strain originally isolated from a rich environment, which did not produce PHB. The experiments showed that the *Spirillum* sp. strain had a higher resistance to nutrient starvation, which correlated to the PHB content, when compared to the *Pseudomonas* sp. strain, giving clues of the

possible association between PHB synthesis and natural environments. Further studies on this subject conclusively showed that PHAs were involved in bacterial survival in unfavorable ecosystems (Handrick, Reinhardt, & Jendrossek, 2000; Kadouri, Jurkevitch, & Okon, 2003; López, Floccari, Steinbüchel, García, & Méndez, 1995).

2.1 Horizontal Acquisition of *pha* Genes

Bacteria living in stressful environments display a wide range of different strategies to deal with the exposure to different challenges. These strategies that endow bacteria with enhanced survival involve the presence of particular sets of genes, many of which can be the result of acquisition through horizontal transfer events (Dobrindt, Hochhut, Hentschel, & Hacker, 2004). The fixation of horizontally transferred genes suggests that they confer a selective advantage on the recipient host. As previously mentioned, the capability to accumulate PHAs has been shown to enhance fitness and survival (Kadouri, Jurkevitch, Okon, & Castro-Sowinski, 2005; López et al., 1995; Ruiz, López, Fernández, & Méndez, 2001; Ruiz, López, & Méndez, 2004). In accordance with this notion, several studies have presented evidence for the acquisition of genes related to PHA biosynthesis by horizontal transfer in different bacterial species. For instance, in *Azotobacter* sp. strain FA8, insertion sequence-like elements belonging to the IS*3* and IS*630* families were found associated with *phb* regulatory genes (Pettinari, Chanetón, Vázquez, Steinbüchel, & Méndez, 2003). Other evidence of this phenomenon was the observation of incongruences between phylogenetic trees constructed using sequences of genes encoding proteins involved in PHA metabolism and trees resulting from 16S rRNA data. These observations supported the idea of the horizontal transfer origin of the *pha* genes in several species. Reports include the anomalous clustering of PhaC of *Azotobacter vinelandii* and *Pseudomonas extremaustralis* (*Pseudomonas* sp. 14-3), both associated with β-Proteobacteria, and probably derived from *Burkholderiales* (Ayub, Pettinari, Méndez, & López, 2007; Kadouri et al., 2005). Moreover, using this approach to individually analyze *phaA*, *phaB*, and *phaC*, horizontal gene transfer of the *pha* genes was proposed in 24 organisms, including both Gram-positive and Gram-negative bacteria (Kalia, Lal, & Cheema, 2007). In *P. extremaustralis*, *phaB* was found to be responsible of the mosaic structure observed in the *phb* cluster (Ayub et al., 2007), and recently the gene encoding the granule-associated protein PhbP of this bacterium was also related to β-Proteobacteria (Catone et al., 2014). Some of these acquired DNA elements have other features such as a different

G + C content, codon bias, and the association with mobility genes, that testify their foreign origin. Among the different types of genetic elements that contribute to the bacterial flexible genetic pool, genomic islands hold a prominent position. It has been reported that the majority of the gene clusters carried by genomic islands encode functions that can be useful for survival and transmission of microbes, thus providing a selective advantage to the island-carrying organism (Hacker & Carniel, 2001). The *phb* genes of *P. extremaustralis*, a microorganism in which the capability to resist multiple stresses has been directly related to the polymer, were found to be in a genomic island (Ayub et al., 2007). More recently, genome analysis of this bacterium allowed the detection of a complete mclPHA gene cluster typical of *Pseudomonas* in the core genome (Catone et al., 2014). When the expression of genes involved in the synthesis of both types of PHAs was assessed, it was observed that *phbC*, encoding the PHB synthase, showed higher expression in comparison with the Pseudomonads core genes encoding mclPHA synthases (i.e., *phaC1* and *phaC2*). This indicates a higher expression efficiency of this gene, presumably acquired by horizontal transfer, that could be related to the high fitness conferred by the capability to produce high amounts of PHB.

2.2 Resistance to Cold

Among the stress factors that impact microbial life, low temperature constitutes a critical issue for growth and survival. Taking into account that cold environments are widespread among the world, low temperature affects a vast number of bacterial habitats. One of the main effects of cold conditions involves the increased production of toxic reactive oxygen species (ROS) that along with physical and biochemical alterations limit bacterial cellular processes (D'Amico, Collins, Marx, Feller, & Gerday, 2006). As indicated before, PHAs are highly reduced carbon and energy storage compounds, and their synthesis is tightly coupled to carbon fluxes and nitrogen metabolism. These polymers are also involved in cellular redox balance, as PHAs operate as a sink for reducing equivalents. Studies performed in *Ralstonia eutropha* (also known as *Cupriavidus necator*) and *Pseudomonas putida*, the model strains for sclPHA and mclPHA production, respectively, have revealed the importance of the intracellular NADH/NAD^+ ratios and the NADPH content in the control of PHA synthesis and depolymerization (De Eugenio, Escapa, et al., 2010; Ren et al., 2009; Schubert, Steinbüchel, & Schlegel, 1988). This, in turn, is in agreement with the importance of PHA to help bacteria cope with oxidative

stress (Ayub, Pettinari, Ruiz, & López, 2004; Ruiz et al., 2004). Regarding the oxidative stress derived from cold, a survival advantage conferred by the capability to accumulate PHB was observed in *P. extremaustralis*, originally isolated from a temporary pond in Antarctica (López et al., 2009). The capability to accumulate PHB was found to be essential to cope with cold, as a PHB synthase mutant (*phaC*) was unable to grow at 10 °C (Ayub, Tribelli, & López, 2009). After a temperature downshift, the level of lipid peroxidation, an indicator of macromolecule damage due to oxidative stress, was 25-fold higher in the mutant strain in comparison with the wild-type strain, in which a rapid degradation of the polymer was observed. Additionally, after the cold shock the NADH/NAD^+ ratio and NADPH content, indicators of the intracellular redox state, decreased noticeably in the mutant in comparison with the wild-type strain. In line with these observations, supplementation of the culture medium with the reducing compounds cystine and glutathione alleviated the cold sensitive phenotype of the *phaC* mutant (Ayub et al., 2009). Antioxidant enzymes (e.g., catalase, superoxide dismutase, and glutathione peroxidase) are known to be induced under cold conditions (Smirnova, Zakirova, & Oktyabrskii, 2001; Zhang et al., 2003), and some of them depend on nicotinamide dinucleotides as cofactors (Cabiscol, Tamarit, & Ros, 2000). In view of this, it has been proposed that the contribution of PHA metabolism to stress resistance could consist in the modulation of the availability of reducing equivalents, thereby contributing to mitigate the oxidative stress resulting from cold exposure (Figure 1).

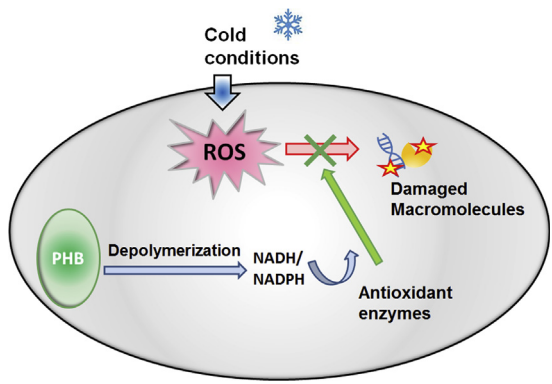

Figure 1 Proposed contribution of poly(3-hydroxybutyrate) (PHB) metabolism in the general bacterial physiology under cold conditions. The absence of PHB could provoke an increase in oxidative stress sensitivity due to a decrease in reducing power availability.

The role of these storage materials in cold adaptation was also observed in other bacterial species. In *Sphingopyxis chilensis*, for instance, PHA has been reported to exert a protective effect against carbon starvation and frozen conditions (Pavez, Castillo, González, & Martínez, 2009), and an increased abundance of PHA synthesis-related enzymes, including PhaP, was observed at low temperature in *Sphingopyxis alaskensis* (Ting et al., 2010). Among other cold-adapted microorganisms that may utilize PHA for their growth is the sea-ice bacterium *Colwellia psychrerythraea* that possesses a significant capacity to produce and degrade fatty acids, providing substrates for mclPHAs biosynthesis (Methé et al., 2005).

Recent studies showed evidence about PHA production in extremely cold environments in the Baltic Sea and Greenland sea ice (Kaartokallio et al., 2013; Pärnänen, Karkman, Virta, Eronen-Rasimus, & Kaartokallio, 2015) by cold-adapted bacteria, where the genes encoding synthases were detected in the bacterial community. This information indicates that the synthesis of these polymers could help bacteria cope with the harsh conditions encountered in such environments. Furthermore, under cold conditions, PHB accumulation was reported to increase motility and survival of planktonic cells in the biofilms developed by *P. extremaustralis*. In view of this, the capability to accumulate PHB could constitute an adaptive advantage for the colonization of new ecological niches in stressful environments (Tribelli & López, 2011).

All this body of evidence strongly supports the link between PHAs and bacterial survival and stress resistance, but what about the mechanisms that endow bacteria with that resistance?

2.3 PHAs and the General Stress Response

As stated before, PHAs are accumulated under conditions of changing nutrient availability. Under nutrient limitation, many bacteria trigger a series of events known as stringent response, that involves guanosine penta- and tetra-phosphates [(p)ppGpp] and other nucleotides. When nutrients are scarce, (p)ppGpp destabilizes the RNA polymerase σ^{70} and therefore reduces the transcription of housekeeping genes, thereby increasing the availability of the polymerase to bind to other σ factors related to stress tolerance, such as σ^{54} or σ^{s} (Magnusson, Farewell, & Nyström, 2005; Potrykus & Cashel, 2008). The σ^{s} (RpoS) factor, encoded by *rpoS*, activates the transcription of genes involved in the bacterial general stress response. Under conditions of high or low temperature, oxidative stress, and low pH, or as the cells transition into the stationary phase of growth, *Escherichia coli* (as well as other

Enterobacteriaceae) and *Pseudomonas* species increase RpoS-dependent gene expression, which leads to general stress resistance (Battesti, Majdalani, & Gottesman, 2011; Hengge, 2009). The first evidence of the relationship between PHAs and these phenomena was obtained analyzing a PHA depolymerization mutant, *P. putida phaZ* (previously referred as *Pseudomonas oleovorans*), providing evidence for an association between PHA degradation and (p)ppGpp accumulation (Ruiz et al., 2001). In this line of experiments, it was later reported that PHA degradation increased stress resistance and the RpoS intracellular levels (Ruiz et al., 2004).

RpoS is also related to other aspects of PHA metabolism. In *A. vinelandii*, the transcription of the *phbBAC* biosynthetic operon is dependent on the σ^s factor (Peralta-Gil, Segura, Guzman, Servín-González, & Espín, 2002), and a role for RpoS in intracellular PHB mobilization in *R. eutropha* has been postulated as well (Brigham, Speth, Rha, & Sinskey, 2012). The authors of this study observed that an *R. eutropha rpoS* mutant displayed all the known traits of this particular genotype but also had increased polymer mobilization when compared to the wild-type strain. A similar phenotype was also observed in a *P. putida* strain deficient in σ^s, thus suggesting that PHA accumulation could help cells to overcome the adverse conditions encountered during the stationary phase (Raiger-Iustman & Ruiz, 2008).

A recent paper (Volova, Zhila, Kalacheva, Brigham, & Sinskey, 2013) describes the synthesis and utilization of PHB in *R. eutropha* grown auxotrophically in cultures supplemented with a nitrogen source. The authors observed that in the PHB utilization phase, *R. eutropha* could not degrade the polymer completely, and they speculated that the reason was that the lack of an external carbon source produced a stress condition that could require the expression of stress genes.

3. GLOBAL REGULATION

Global regulatory networks allow bacteria to control multiple cellular functions in response to environmental changes. Transcription factors control genes and operons that belong to different metabolic pathways (Ishihama, 2010) and small RNAs (sRNAs) regulate gene expression by binding mRNA or proteins (Gottesman & Storz, 2011). The role of both types of regulators on PHA production is just beginning to be unveiled, providing explanations for the effect of the cellular physiological status on the synthesis and degradation of the polymer.

3.1 The Stringent Response

PHAs accumulate under unbalanced growth conditions induced, among other factors, by the lack of a nitrogen source, thus linking PHA accumulation to the stringent response. The proteins involved in the synthesis of (p)ppGpp in *E. coli* are RelA, which activates the nucleotide synthesis under amino acid starvation, and SpoT, which increases (p)ppGpp intracellular levels in response to other nutrient stresses (Potrykus & Cashel, 2008). A recent paper reported that NtrC, the regulator of nitrogen assimilation in *E. coli*, stimulates *relA* transcription in cultures subjected to nitrogen-limiting conditions (Brown, Barton, Pan, Buck, & Wigneshweraraj, 2014).

Genome sequencing has revealed genes homologous to *relA* in different bacterial strains, such as *spoT2* in *R. eutropha* and *rsh* in Rhizobia. The relationship between PHA accumulation and the stringent response remained almost unnoticed in the work of Calderón-Flores et al. (2005), who only mentioned the PHB-negative phenotype of a *rsh* mutant of *Rhizobium etli* in the legend of a figure.

A complete analysis later demonstrated, by means of genome-wide DNA microarrays and the study of several mutants, that PHA synthesis is regulated by the stringent response (Brigham, Speth, et al., 2012). The experiments were done in *R. eutropha,* the model organism for PHB production. In this bacterial species, the structural genes for the synthesis and degradation of the polymer encode a β-ketothiolase (PhaA), an acetoacetyl-coenzyme A (CoA) reductase (PhaB), a PHB polymerase (PhaC), and a PHB depolymerase (PhaZ). The transcriptional regulation of these genes in this microorganism is provided by the regulator PhaR, and a structural phasin protein, PhaP1, is also present (Pötter, Madkour, Mayer, & Steinbüchel, 2002; York, Stubbe, & Sinskey, 2002). Analysis of global gene expression during PHB synthesis in *R. eutropha* cultures subjected to nitrogen depletion clearly showed an enhanced transcription of genes regulated by σ^{54}, and downregulation of the housekeeping genes compared to control cultures, nonrestricted in the nitrogen source. Furthermore, mutants impaired in (p)ppGpp synthesis are unable to accumulate significant amounts of PHB.

On a previous study performed with a mixed consortia fed with wastewater, the PHA concentration correlated statistically with the content of (p)ppGpp in the biomass (Al-Najjar, Coats, & Loge, 2011). These results, together with the above-mentioned demonstration that PHA degradation in *P. oleovorans* was associated with ppGpp accumulation

(Ruiz et al., 2001), led the authors to propose that the stringent response regulates the cycle of PHA production.

3.2 Catabolite Repression

Catabolite repression controls carbon metabolism regulating the hierarchical and sequential use of the carbon sources present at nongrowth-limiting concentrations in the medium. The Crc (*c*atabolite *r*epression *c*ontrol) protein is a key regulator involved in the repression by catabolites in *Pseudomonas*. It acts at the translational level through the interaction with a specific sequence, A*n*AA*n*AA (where *n* represents any nucleotide), located near the ribosome binding site in the target mRNAs (Moreno, Marzi, Romby, & Rojo, 2009; Sonnleitner, Abdou, & Haas, 2009). The Crc protein level is controlled by the sRNA CrcZ in *Pseudomonas aeruginosa* (Sonnleitner et al., 2009), and by two sRNAs (CrcZ and CrcY) in *P. putida*. Catabolite repression is strong during the exponential phase of growth, but at the stationary phase the high-level concentration of CrcZ and CrcY sequesters Crc and allows the translation of the repressed mRNA (Moreno, Fonseca, & Rojo, 2012).

Hfq is a protein involved in posttranscriptional regulation, including the one mediated by Crc (Gottesman & Storz, 2015). A model for the cooperative action of Crc and Hfq, in which both proteins co-interact with the particular mRNA motif, has been recently proposed in *P. putida* (Moreno et al., 2014). The authors also suggested that CrcZ and CrcY do not regulate Hfq levels.

As PHAs are carbon reservoirs, their synthesis and degradation are expected to be affected by catabolite repression. A recent work performed in *P. putida* KT2440 addressed this subject (La Rosa, Peña, Prieto, & Rojo, 2014). In this strain, *phaC1* and *phaC2* encode two PHA polymerases, and *phaZ* encodes a PHA depolymerase. Other genes involved in PHA synthesis and degradation are *phaF* and *phaI*, responsible for the synthesis of phasin and a structural protein, respectively, while *phaD* encodes a positive transcriptional regulator (De Eugenio, Galán, et al., 2010; Prieto, De Eugenio, Galán, Luengo, & Witholt, 2007). The canonical Crc sequence differing in the *n* nucleotide was found close to the ribosome binding site in *phaC1*, *phaF*, and *phaI*. However, Crc only inhibited the translation of the *phaC1* mRNA, and the expression of neither *phaF* nor *phaI* was affected by the regulator. In addition, the action of Crc was limited to the exponential phase of growth, and it was not observed when the cultures entered into the stationary phase, in which PHA accumulation reached its maximum due to the antagonism, as explained before, of the sRNAs CrcZ and CrcY.

3.3 Control by the Signal Transduction Pair GacS/GacA

PHA synthesis is also affected by another global regulator system, GacS/GacA (*g*lobal *a*ntibiotic and *c*yanide control), that also regulates alginate biosynthesis. GacS is a sensor and GacA is its cognate regulator that activates the expression of sRNAs called RsmX/Y/Z. These sRNAs, together with RsmA, constitute a regulatory system in which RsmA inhibits the translation by binding to specific sequences in mRNAs, whereas the sRNAs bind to RsmA repressing its regulatory activity. This system is therefore called Gac/Rsm.

In *A. vinelandii*, genes involved in PHB synthesis are organized in the *phbBAC* operon that encodes an acetoacetyl-CoA reductase, a β-ketothiolase, and a PHB synthase, respectively. These genes are controlled by a regulator encoded by *phbR*. The regulatory effect of GacS on PHB synthesis in *A. vinelandii* was observed in *gacS* mutants (Castañeda, Guzmán, Moreno, & Espín, 2000) and further analyzed by means of in vitro assays and the expression of transcriptional and translational fusions to *phbR*. It was concluded that GacA enhances the synthesis of eight sRNAs present in the *A. vinelandii* genome, and that RsmA controls PhbR at the translational level, and as a consequence, the PHB synthesis in this species (Hernández-Eligio et al., 2012).

A different kind of control exerted by GacS/GacA on PHA synthesis was recently described in *P. putida* CA-3 (Ryan, O'Leary, O'Mahony, & Dobson, 2013). Gac/Rsm-regulated genes in this strain share 96% similarity with those in the *P. putida* published genome. However, a *gacS* mutation obtained by random Tn*5* mutagenesis prevented the translation of the *phaC1* mRNA, even if it did not affect RsmY and RsmZ transcription, suggesting another pathway of Gac/Rsm regulation of PHA synthesis.

3.4 Redox Regulation

FNR (*f*umarate and *n*itrate *r*eduction) is a global regulator that controls the transcription of genes encoding functions that facilitate adaptation to growth under oxygen-limiting conditions in *E. coli* (Crack, Green, & Thomson, 2004). The global regulator CydR is an Fnr-like protein which is known to regulate the expression of the cytochrome genes *cydAB* in *A. vinelandii*. A *cydR* null mutant of this species was found to have diminished expression of the genes encoding fumarase C and CoA transferase, and also showed enhanced PHB synthesis. These effects were only observed during the exponential phase of growth, while at the beginning of the stationary phase the

wild-type strain produced higher amounts of polymer than the mutant. The authors explained this phenotype by the enhanced expression of the genes encoding the β-ketothiolase (PhbA) and acetoacetyl-CoA reductase (PhbB) through the exponential growth phase (Wu, Moir, Sawers, Hill, & Poole, 2001).

In *Pseudomonas*, a transcriptional regulator highly homologous to Fnr in *E. coli*, named Anr (*a*rginine and *n*itrate *r*eduction), is known to control a set of genes involved in the aerobic-to-anaerobic transition, encoding, among others, nitrate respiration functions, arginine fermentation, hydrogen cyanide, and heme synthesis in anaerobiosis (Follonier et al., 2013; Galimand, Gamper, Zimmermann, & Haas, 1991; McPhee et al., 2009; Zimmermann, Reimmann, Galimand, & Haas, 1991). A DNA microarray analysis performed in *P. putida* under conditions of elevated pressure and oxygen availability indicated a decrease in the transcription of *anr* and, concomitantly, a low level of the transcripts corresponding to terminal oxidases (Follonier et al., 2013). In *P. extremaustralis*, a bacterium highly resistant to stress which produces PHB and also PHAs of different monomeric composition, the effect of Anr on PHB synthesis was analyzed by means of an *anr* mutant under different oxygen availability conditions. The mutation prevented bacterial growth in anaerobic conditions with nitrate as terminal electron acceptor, while PHB synthesis in microaerobic and aerobic conditions was lower compared to the wild-type strain, and associated with a lower expression of *phaC* and *phaR* genes. These results suggested that Anr is involved in the regulation of *pha* genes (Tribelli, Méndez, & López, 2010).

The two-component signal transduction **s**ystem ArcAB modulates the transcription of many operons according to the redox state of the environment. ArcB is a transmembrane sensor kinase and ArcA is the cognate response regulator. Under microaerobic and anaerobic conditions, the main targets for repression of this system are the genes that encode the enzymes involved in aerobic respiration and those of the tricarboxylic acid (TCA) cycle (Lynch & Lin, 1996). Consequently, the *arcA* mutants are unregulated for aerobic respiration functions, and the genes encoding components of the TCA cycle are fully expressed under microaerobic growth conditions. The effects of this mutation on PHA synthesis were assessed in an *E. coli* recombinant strain hosting *pha* genes from *Azotobacter* sp. strain FA8 (Pettinari et al., 2001). PHB synthesis was not detected in the wild-type strain in shaken-flask cultures under low-oxygen conditions, while *arcA* mutants gave rise to polymer accumulation up to 24% (w/w) of the cell dry weight (CDW) (Nikel, Pettinari, Galvagno, & Méndez, 2006). It

was concluded that ArcAB exerted an indirect (metabolic) regulation on PHB synthesis in the recombinants, through the de-repression of the genes involved in aerobic respiration, thus increasing the availability of reducing power.

3.5 The PTS System

An unexpected regulation of PHA accumulation is that exerted by some components of the PTS phosphotransferase system (Velázquez, Pflüger, Cases, De Eugenio, & de Lorenzo, 2007). PTS mediates the flow of high-energy phosphate from phosphoenolpyruvate to transported sugars. The genome of *P. putida* KT2440 presents five PTS genes: *fruA*, *fruB*, *ptsO*, *ptsP*, and *ptsN*. While the products of *fruA* and *fruB* are involved in fructose uptake, the other genes seem to be involved in unexpected functions, as mutations in each of them produced different growth phenotypes related to nitrogen sources when compared to the parental strain. Previous work showed that inactivation of *ptsP* affects PHB accumulation in *A. vinelandii* (Segura & Espín, 1998), so the synthesis of PHA by *P. putida* was analyzed to investigate these phenotypes. This strategy revealed that mutations in *ptsN* are sensed by the PHA synthesis apparatus as an abundance of carbon relative to other nutrients, while the absence of PtsP and PtsO would send the opposite signal. Thus, the components of the PTS system could function by gauging the balance between carbon and nitrogen sources and, as a consequence, they ultimately affect PHA accumulation.

4. PHA ACCUMULATION AND COORDINATED REGULATION OF CENTRAL METABOLISM

In spite of the increasingly broad understanding of the role of metabolic intermediates and precursors, distribution of carbon fluxes, and redox potentials on PHA accumulation in several bacterial species, relatively little is known about the integration of PHA synthesis in the bacterial metabolism. In particular, the central question of how PHA accumulation impacts the overall physiology of the bacterial cell remains largely unanswered. The question posed here can be tackled from two different (and complementary) perspectives. Firstly, recombinant *E. coli* strains are considered a suitable model to study the impact of polymer accumulation on the bacterial cell physiology since the host does not have the enzymes needed for the synthesis or the degradation of PHAs (Leong et al., 2014). On the other hand, in natural producer microorganisms such as Pseudomonads, the metabolism of PHAs is

involved in a complex and tightly regulated cycle of synthesis and degradation that lies at the very core of the bacterial central carbon catabolism (Luengo, García, Sandoval, Naharro, & Olivera, 2003; Nikel, Martínez-García, & de Lorenzo, 2014). In the latter example, the interactions between PHA formation and hydrolysis and central metabolism can be interpreted as the final consequence of the different levels of metabolic regulation (i.e., transcriptional, translational, and enzymatic). Below we present some examples of studies devoted to elucidate these features in both recombinant *E. coli* strains and *Pseudomonas* species.

4.1 PHA Accumulation in Recombinant *Escherichia coli* Strains: Metabolic Flexibility and Global Regulation of Carbon and Redox Balances

The heterologous PHB synthesis in *E. coli* elicits a number of global regulatory and metabolic responses, a well-established fact supported by several lines of experimental evidence. Most of these regulatory alterations have been explored at the transcriptional level, but information on how PHB synthesis is integrated into the central biochemical network of the bacterium is relatively limited. Exploring these features is, however, a relevant task for metabolic engineers, as the resulting information could provide clues on possible strategies to improve the overall output of PHA-producing processes. For the sake of the present review, in this section we will focus on the recent advances regarding the physiological consequences of PHA formation in *E. coli* recombinants. For instance, Sekar and Tyo (2015) recently used controlled, steady-state chemostat cultures to determine growth-independent regulation patterns on central metabolic fluxes in a recombinant *E. coli* accumulating PHB. They employed a series of recombinant strains in which the *phaCAB* genes from *R. eutropha* have been integrated into the host bacterial chromosome in different copy numbers. Fluxes and steady-state intracellular metabolite concentrations were measured across different dilution rates ($D = 0.05$, 0.15, and 0.30 h^{-1}), nutrient limitations (glucose, gluconate, and nitrogen), and number of copies of the *pha* operon (0, 6, 17, and 29 copies). As the PHB formation flux increased in nitrogen-limited, glucose-fed conditions, so did the specific substrate consumption rate and lactate secretion (formed to maintain redox balance), while the specific rates of formate and acetate secretion decreased.

As these experiments clearly showed that PHB accumulation altered the fate of carbon and reducing equivalents, a flux balance analysis model was applied to the data to further analyze intracellular redox and energy

conditions. Most surprisingly, the results of such model suggested that, under these specific nitrogen-limited conditions, PHB formation results in a net formation of reducing equivalents—which the cells spill off as lactate. The cells hence secreted more reduced metabolites to recycle reducing equivalents. Following this in silico evidence, the authors reasoned that by switching the fed to a more oxidized substrate (i.e., gluconate), the metabolism of which generates less reducing equivalents than that of glucose, PHB formation should increase. Indeed, the PHB formation flux in gluconate cultures increased 1.6-fold (up to 1.2 mmol/g_{CDW}/h) compared to those in glucose-fed fermentations. These results are in full agreement with the evidence gathered by Nikel, de Almeida, Giordano, and Pettinari (2010), showing that the amount of PHB accumulated by *E. coli* recombinants can be altered by selecting carbon sources differing in their degree of oxidation.

Wlaschin, Trinh, Carlson, and Srienc (2006) followed an integrated approach to investigate the impact of PHB accumulation on the physiology of recombinant *E. coli* under anoxic conditions. The main idea here was to decompose an intricate metabolic network comprised of highly interconnected reactions into uniquely organized (and simpler) pathways—thereby providing an integrated view of the impact of any perturbation in central metabolism. Since the entire metabolism of a cell can be viewed as a weighted sum of elementary modes (i.e., metabolic contributions to a given pathway), the authors tried to identify the individual weights of each node, a nontrivial problem considering the multiplicity of metabolic modes in a cell (even more so in a recombinant strain). To enable the determination of weighting factors, two gene deletions (*ldhA*, encoding lactate dehydrogenase, and *frdA*, encoding fumarate reductase) were combined with anoxic growth conditions to limit the metabolism from 4374 original elementary modes to just 24 elementary modes (in a strain that does not accumulate PHB) or 40 elementary modes (in a PHB synthesizing strain). These modes were grouped into five families that have the same overall stoichiometry, and the weighting factors for each family of modes were determined from the measurement of accumulation rates of selected metabolites. The authors found that individual weights were inversely correlated with the entropy generated by the operation of the used pathways defined in elementary modes. Such correlation provides a rational way of studying the regulation of metabolic fluxes based on the thermodynamic properties of elementary modes. Under nongrowing conditions, the metabolic network of a cell collects the chemical bond energy from high-enthalpy, low-entropy ordered substrates, by converting them into low-enthalpy, high-entropy products.

This experimental evidence suggests an important evolutionary rule governing the organization of central metabolism. Evolution seems to have developed regulatory patterns that permit diversity of pathways while favoring efficient pathways with low entropy generation—a basic principle that also applies for the evolution of the PHA biosynthetic pathway. This feature is a common principle that can be exploited to understand the relationship between the accumulation of an heterologous product and the core metabolic network of the host.

From the evidence presented in the examples above, the immediate conclusion is that *E. coli* can accommodate the metabolic load of heterologous PHA formation by readapting some key fluxes in the entire biochemical network in such a way that carbon and reducing equivalents are efficiently recycled. In particular, these studies have conclusively shown that the fermentation pathways of *E. coli* are the preferred metabolic "security valves" used to efficiently dump off reducing equivalents when global carbon and redox balances are altered (e.g., under PHB synthesizing conditions). Yet, how does the cycle of PHA accumulation and degradation interact with other cellular processes in a natural producer?

4.2 Metabolic Regulation and PHA Accumulation in Natural Producer Bacteria

The impact of PHA metabolism on the central carbon catabolism has been quantitatively studied in *P. putida* KT2442. A key factor known to influence polymer formation in Pseudomonads is the availability of NAD(P)H, as a high NAD(P)H/NAD(P)$^+$ ratio has been found to inhibit the entry of acetyl-CoA in the TCA cycle, thereby stimulating PHA synthesis (Haywood, Anderson, Chu, & Dawes, 1988). Escapa, García, Bühler, Blank, and Prieto (2012) have systematically analyzed PHA production through quantitative physiology experiments in a wild-type strain and in a PHA-negative mutant derivative growing under nitrogen-limited conditions. In these experiments, the PHA-negative mutant bore a *phaC1*::mini-Tn*5* insertion, which rendered the PhaC1 polymerase inactive. Octanoic acid, which serves as a PHA precursor in Pseudomonads, was used as the sole carbon and energy source throughout this study. Higher intracellular fluxes to acetyl-CoA were detected in the mutant strain when compared to wild-type KT2440, suggesting a role of PHA formation in imposing a balance in the distribution of central carbon fluxes. This metabolic feature was accompanied by the transcriptional activation of genes encoding components of the TCA cycle and the glyoxylate shunt, as observed in genome-wide DNA array experiments.

The activation of these metabolic pathways would in turn redirect the excess of acetyl-CoA into the lower catabolism. One key finding of this work was that in the *phaC1* mutant, negatively affected in the pathways needed for efficient carbon and energy storage, carbon spills off as carbon dioxide due to an increase in respiration, instead of generating more biomass as it might have been anticipated. Acetate was also secreted to the extracellular medium as an overflow metabolite in cultures of the *phaC1* mutant. The authors concluded that *P. putida* operates its central metabolic pathways to optimally exploit the available resources, channeling excess carbon and reducing equivalents to storage compounds (through the accumulation of PHA) but without compromising growth. These findings demonstrate that PHA metabolism in Pseudomonads plays a critical role in synchronizing the activity of central metabolic pathways with the availability of nutritional resources.

The physiological responses of *P. putida* KT2442 to PHA accumulation were also systematically investigated by using a multi-omic approach to highlight metabolic differences between single- and multiple-nutrient-limited growth in chemostat cultures (Poblete-Castro et al., 2012). The dilution rate was set at $D = 0.1\ h^{-1}$ to ensure PHA formation, and decanoate and NH_4Cl were used as the sole carbon and nitrogen sources. The authors reported that 26%, 62%, and 81% of the CDW consisted of PHA under conditions of carbon, carbon and nitrogen, and nitrogen limitation, respectively. Interestingly, the highest specific PHA production rate, $0.43\ g_{PHA}/g_{CDW} \cdot h$ was obtained under nitrogen limitation. The residual biomass was not constant for dual- and strict nitrogen-limiting growth, which sets strain KT2442 apart from other *P. putida* strains, in which biomass formation remains a constant among different nutrient limitations. In line with these observations, dual-limitation continuous cultures resulted in patterns of gene expression, protein level, and metabolite concentrations that substantially differed from those observed under exclusive carbon or nitrogen limitation. The most pronounced differences were found in the energy metabolism, fatty acid metabolism, as well as stress proteins and enzymes belonging to the transport system. One striking difference, detected in the nitrogen and dual-limited chemostat when compared with carbon limitation, was a sharp increase in the transcription of genes encoding the branched-chain amino acid ABC transporter. The same held true for *phaI* and *phaF*, encoding two granule-associated proteins, and *phaC1* and *phaC2*, encoding two PHA synthases. The expression of porins under both nitrogen and dual limitation correlated well with the uptake of the carbon source, indicating that the cells adjust the input of carbon in response to

nutrient limitation to ensure sufficient carbon needed for each cellular process. The TCA cycle was found to be repressed under dual nutrient limitation through a carbon catabolite mechanism, probably mediated by the NtrC global regulator. Genes and proteins involved in ATP generation through the respiratory chain were found to be overexpressed as the amount of PHA accumulated increased, indicating that cells forming PHA could undergo a shortage in their energy resources. This study clearly demonstrated that PHA formation and hydrolysis affect a number of cellular processes in strain KT2442, and not only, as initially thought, those strictly related to carbon and nitrogen metabolism.

A study that also deserves mention, as it offers an innovative technical approach, is that of Nikodinovic-Runic, Flanagan, Hume, Cagney, and O'Connor (2009). The authors adopted a systematic proteomic approach to interrogate *P. putida* CA-3 on the changes brought about by PHA accumulation from styrene. Besides the interesting physiological question on PHA biosynthesis regulation, this study provides an insight on the formation of bioplastics from styrene, an industrially relevant conversion of a nonbiodegradable polymer (i.e., polystyrene) into an environmentally friendly, entirely biodegradable one (i.e., PHA). The strain studied is a styrene-degrading bacterium which synthesizes PHA when exposed to nitrogen-limiting conditions. Using shotgun proteomics, the authors analyzed global proteome expression in cultures of *P. putida* CA-3 supplied with styrene as the sole carbon and energy source under nitrogen-limiting and nonlimiting growth conditions. A total of 1761 proteins, belonging to diverse functional groups, such as styrene degradation, energy balance, nucleotide metabolism, protein synthesis, transport processes, stress responses, and motility, were identified with a high level of confidence. Expectedly, most of the proteins involved in the upper and lower styrene degradation pathway were expressed irrespective of the growth condition regarding nitrogen availability. Proteins related to PHA accumulation and biosynthesis were only expressed under nitrogen limitation, and nitrogen assimilation proteins were detected on average at twofold higher amounts under nitrogen limitation. Interestingly, the uptake of branched chain amino acids by cells grown in nitrogen-limited cultures was higher than that by cells grown in nonlimited cultures. Again, the expression of the branched-chain amino acid ABC transporter was up to 16-fold higher under nitrogen-limiting conditions, suggesting a tight coordination between nitrogen metabolism (both at the level of transport and assimilation) and polymer formation.

The examples above illustrate how central the metabolism of PHA is in natural producer bacteria, affecting a number of cellular functions far beyond the regulation of nutrient availability. The omic approaches discussed in these studies exposed the role of PHA as a regulator of features as diverse as stress responses and cellular motility. However, further systems-level studies are still needed to understand how these responses are integrated in a coordinated fashion—revealing, at the same time, strategies for targeted metabolic engineering manipulations.

5. ENVIRONMENTAL APPLICATIONS OF PHA-PRODUCING ORGANISMS

Based on the information available at present, there is no doubt that PHAs play a key role in the physiology of natural producers and are essential for survival in some environments, also having a high ecological relevance. Knowledge of the benefits conferred by the polymer may be of interest for various biotechnological applications that use bacteria to solve environmental problems. The rationale of some of these applications will be briefly commented in this section.

5.1 Bioremediation

Oil-contaminated places are stressful environments due to toxicity of pollutants and also by the oxidative stress generated by these compounds. Bioremediation strategies designed to remove pollutants from these contaminated sites use organisms that must be able to adapt to stresses prevailing in the environment (Tyagi, da Fonseca, & de Carvalho, 2011). Thus, the presence of some intrinsic mechanisms to cope with those stresses, such as the capability to accumulate PHA, is a desirable characteristic. PHAs can be produced from different carbon sources, including different hydrocarbons such as benzene, toluene, and xylene (BTX) (De Smet, Eggink, Witholt, Kingma, & Wynberg, 1983; Ni et al., 2010; Nikodinovic et al., 2008; Prieto, 2007); thus, the same compound that is targeted for elimination could be used for the synthesis of the polymer contributing to the persistence of the bioremediation agent in the environment and, consequently, the overall efficiency of the process. The capability to grow using several hydrocarbons as sole carbon source and to accumulate PHAs of two *Pseudomonas* strains (KA-08 and KB-08) isolated from an oil refinery wastewater were analyzed (Di Martino, López, & Raiger-Iustman, 2012). Apart from the capability to use different hydrocarbons and to degrade and tolerate high

concentrations of benzene, toluene, and xylenes (BTX), both strains were able to synthesize surfactant compounds. One of the problems concerning oil bioremediation is the hydrophobic nature of oil derivatives that decreases their availability as carbon sources. A possible way to enhance their bioavailability and, thereby, their biodegradation, is the presence of surfactants. Many bacterial species, such as most of the alkane-degrading bacteria, can synthesize these compounds that help to increase hydrocarbon accessibility (Ron & Rosenberg, 2002). The metabolism of PHAs and some biosurfactant compounds are related. Degradation of PHAs releases 3-hydroxyacyl-CoA that could be used for the synthesis of biosurfactants, such as rhamnolipids produced in *P. aeruginosa* (Soberón-Chávez, Aguirre-Ramírez, & Sánchez, 2005). Selection of bacteria with the capability to tolerate and degrade monoaromatic compounds and synthesize biosurfactants and PHAs, that enhance stress resistance, could be a good approach for efficient bioremediation strategies (Di Martino et al., 2012). It has been reported that the capability to synthesize PHAs had a favorable effect on the production of compounds that affects surface tension, but not on the production of bioemulsifiers, another important trait for hydrocarbon remediation. On the other hand, PHA accumulation affects the cellular affinity to hydrocarbons, as it was observed that PHA-negative mutants of *Pseudomonas* sp. KA-08 and *P. putida* GPp104 have increased affinity to xylene when compared to the wild-type strain (Di Martino, Catone, López, & Raiger-Iustman, 2014). This suggests that manipulation of PHA accumulation conditions could be useful to control surfactant and bioemulsifier activity in hydrocarbon-contaminated sites.

The effect of PHA accumulation capability on bioremediation was studied in *P. extremaustralis*, also considering the protected environment encountered in biofilms. The influence of cell growth conditions (including differences in PHA accumulation) on diesel degradation was analyzed in biofilm and planktonic cultures of this bacterium. Biofilms showed increased cell growth, biosurfactant production, and diesel degradation compared with those obtained in shaken-flask cultures. PHA accumulation decreased biofilm cell attachment and enhanced biosurfactant production (Tribelli, Di Martino, López, & Raiger-Iustman, 2012). These results are in accordance with those indicating that in planktonic- and biofilm-grown *P. aeruginosa*, PHA biosynthesis genes are differentially regulated (Campisano, Overhage, & Rehm, 2008).

An interesting application based on the ecological role of PHAs involves the treatment of wastewaters. PHA is produced by many bacteria

under dynamic conditions of synthesis and degradation (De Eugenio, Escapa, et al., 2010). In wastewater treatment processes, storage of PHAs by mixed populations occurs under transient conditions derived from the exposure to an effective selective pressure caused by discontinuous feeding, such as the application of a cyclic change in feast and famine conditions, or a variation in the electron donor/acceptor presence (Johnson, Jiang, Kleerebezem, Muyzer, & van Loosdrecht, 2009; Reis et al., 2003; Rodgers & Wu, 2010). These aerobic feast/famine processes are suitable for the treatment of wastewaters with excess organic carbon and low nitrogen and phosphorus content, while anaerobic—aerobic dynamic processes are adequate for the treatment of wastewaters containing high carbon and phosphorus concentrations. In addition to PHA accumulation, these processes involve the dynamic accumulation of glycogen, a carbon polymer that has a metabolism related to that of PHA (further discussed in Section 6.2. below), and polyphosphate. Thus, the application of ecological selection principles allows the survival and the enrichment of PHA producers through the cyclic change in the environmental conditions. This strategy, based on natural selection and competition, has relevance in wastewater treatment as well as in potential processes for PHA production and polyphosphate recovery.

5.2 Plant Growth Promotion

The ecophysiology of PHA production could be also useful for agricultural applications. Recently, PHB accumulation has been identified as a key physiological property in *Azospirillum brasilense*, contributing to rhizosphere adaptation and plant growth promotion abilities (Fibach-Paldi, Burdman, & Okon, 2012). Several studies using genetic approaches based on mutant construction have demonstrated that PHAs are major determinants for overcoming periods of carbon and energy starvation and to tolerate and survive to several stresses, such as UV-irradiation, heat, osmotic shock, desiccation, and oxidative stress in this bacterium (Kadouri et al., 2003; Kadouri et al., 2005). In addition, polymer production appears to be an important trait for root colonization and plant growth promotion, as inoculants prepared with PHA-rich *Azospirillum* cells showed increased crop yields (Dobbelaere et al., 2001). In symbiotic nitrogen-fixing bacteria, the invasion of plant tissues involves the exposure to stressful conditions; so the capability to accumulate PHAs can provide metabolic advantages to the bacteria in these circumstances (Trainer & Charles, 2006).

Several studies have shown that PHB is required for successful competitiveness in the nodulation process in different species of rhizobia (Aneja, Zachertowska, & Charles, 2005; Cevallos, Encarnación, Leija, Mora, & Mora, 1996; Quelas, Mongiardini, Pérez-Giménez, Parisi, & Lodeiro, 2013). The knowledge of the relevance of PHA accumulation on bacterial survival under unfavorable conditions could contribute to improve the performance of inoculants that promote plant growth.

6. RELATIONSHIP OF PHAs WITH OTHER POLYMERS AND EXTRACELLULAR SUBSTANCES

PHAs are key players in the metabolism of many bacteria, involved in cell homeostasis through different functional roles. As mentioned before, PHA accumulation allows cells to dispose of excess carbon and reducing equivalents and to have these resources readily available when needed for different cellular processes (Escapa et al., 2012). However, PHAs are not the only polymers accumulated by bacteria. Many of them can accumulate other intracellular polymers, such as glycogen, and also many types of extracellular compounds, collectively denominated extracellular polymeric substances (EPS). Most commonly, bacterial EPS are represented by polysaccharides such as alginate (Rehm, 2010).

6.1 PHAs and Extracellular Substances

Although many of these compounds compete for biosynthetic precursors and can be used by microbial cells for similar purposes, they are normally produced in different moments during the cell cycle, responding to diverse environmental conditions. For example, the analysis of the production of EPS and PHA in *Azotobacter beijerinckii* (Pal, Manna, & Paul, 1999) and in *R. eutropha* (Wang & Yu, 2007) showed that, while EPS synthesis basically accompanied growth and was strongly stimulated by nitrogen availability, PHA accumulation was favored by cell growth restrictive conditions, including nitrogen limitation.

Bacterial biofilms are complex structures in which microorganisms are embedded in a matrix and attached to a surface. This lifestyle represents a protected mode of growth that allows cells to survive in unfavorable environments, to cope with stress, and to disperse, colonizing new ecological niches (Decho, 2000). It is now known that they are the preferred lifestyle in natural and artificial environments. The attachment of microbial cells to a surface is a complex process influenced by several factors, including the

metabolism of carbon polymers (Donlan, 2002) that are key components of the extracellular matrix. This matrix includes mainly exopolysaccharides, like alginate, but it has been reported that other compounds, such as rhamnolipids, play a major role in the architecture of biofilms produced by *P. aeruginosa* (Davey, Caiazza, & O'Toole, 2003). Metabolism of these compounds and PHAs seem to be related both by sharing intermediate molecules for their biosynthesis and also by competing for the cellular carbon fluxes. Apart from their role in biofilm formation, rhamnolipids are involved in the uptake of hydrophobic substrates, virulence, resistance to antimicrobials, and motility (Abdel-Mawgoud, Lépine, & Déziel, 2010). The synthesis of these compounds is catalyzed by rhamnosyltransferase 1, composed of the proteins RhlA and RhlB. In *P. aeruginosa*, RhlA has been observed to affect PHA biosynthesis regulation (Soberón-Chávez et al., 2005). Both compounds are composed of 3-hydroxydecanoic acids connected by ester bonds, competing for fatty acid precursors, so it was not surprising to observe that RhlA mutants, unable to synthesize rhamnolipids, accumulated more PHAs. Unexpectedly, plasmid overexpression of *rhlA* in the *rhlA* mutant did not restore rhamnolipids synthesis, but resulted in increased PHA accumulation, leading the authors to propose that this enzyme is involved in the synthesis of PHA precursors, and affects the distribution of fatty acid derivatives among these compounds (Soberón-Chávez et al., 2005). Another study conducted in *P. aeruginosa* investigated the relationship between rhamnolipids, PHAs, and another polymer, alginate, also involved in the production of the biofilm matrix (Pham, Webb, & Rehm, 2004). In this work, elimination of PHA synthesis was observed to decrease rhamnolipid synthesis but increase alginate production in the wild-type strain, while in an alginate over-producing strain this polymer was unaffected and rhamnolipid production was increased by eliminating PHA biosynthesis, thus suggesting that PHA biosynthesis and alginate biosynthesis were in competition for a common metabolic precursor. A similar result was observed in a PHB mutant strain of *P. extremaustralis* in biofilms developed under cold conditions, where the defect in PHB production resulted in an increase of EPS, suggesting that carbon molecules availability could redirect PHB production to other carbon polymers such as EPS (Tribelli & López, 2011). Interestingly, the regulation of genes involved in PHA biosynthesis in biofilms showed a spatial distribution similar to that of rhamnolipid biosynthesis genes (Campisano, Overhage, & Rehm, 2008).

Other studies, performed in *A. vinelandii*, another good alginate producer, also showed increased alginate synthesis in mutants unable to synthesize

PHAs, and augmented PHA synthesis in mutants impaired in alginate production (Segura, Guzmán, & Espín, 2003). On the other hand, GacA insertion mutants have decreased synthesis of both alginate and PHB (Castañeda, Sánchez, Moreno, Núñez, & Espín, 2001), suggesting a common regulation. However, this mutant has many pleiotropic effects (expectedly, since GacA is a global regulator that affects multiple functions) including the production of many secondary metabolites (Reimman et al., 1997), so the observed phenotype may not indicate a direct relationship between both polymers. However, another study performed in *Sinorhizobium meliloti* showed that elimination of PHA synthase resulted in the abolishment of the production of both PHA and the EPS succinoglycan, suggesting that the synthesis of both polymers share regulation steps (Aneja, Dai, Lacorre, Pillon, & Charles, 2004). Furthermore, studies that analyzed the role of different polymers in symbiotic relationships of *S. meliloti* proposed that PHB could promote EPS synthesis during invasion, thus increasing the rate and efficiency of nodule occupancy (Wang et al., 2007).

These results suggest complex interactions between the biosynthetic pathways of PHAs and extracellular substances, such as EPS, that can differ between microorganisms, and reflect that there are still many unknown aspects concerning the way that bacteria manage synthesis and degradation of these compounds according to their physiological needs.

6.2 PHAs and Glycogen

The role of PHAs in survival to nutrient starvation is well documented (Kadouri et al., 2005), and this characteristic is used to enrich mixed cultures in PHA producers by alternating feast and famine cycles (Reis et al., 2003). Glycogen is, together with PHAs, the most widespread intracellular polymer in bacteria. Glycogen is accumulated by both eukaryotic and prokaryotic microorganisms and acts as a carbon and energy reserve polymer. However, in spite of the fact that (1) many microorganisms accumulate both PHAs and glycogen, (2) glycogen has also been reported to accumulate under growth-limiting conditions such as nitrogen limitation (Schwarz & Forchhammer, 2005; Zevenhuizen, 1981), and (3) these intracellular polymers seem to have similar functions (Lodwig et al., 2005), the relationship between them has not been extensively studied. Both are produced when carbon is present in excess, and they can compete for metabolic precursors. In view of this, it is not surprising that PHA synthase mutants accumulate increased amounts of glycogen, as reported for *R. etli* (Cevallos et al., 1996) and for *S. meliloti* (Wang et al., 2007). However,

the few studies that have analyzed the dynamics involved in the synthesis and use of these polymers in the cells have observed differences (Monshupanee & Incharoensakdi, 2014) suggesting that the roles of these compounds in the cell physiology are not the same. For example, while both polymers are synthesized from acetyl-CoA and have been proposed to act as carbon sinks, their elimination has different metabolic consequences. In *Synechocystis*, mutants impaired in PHA synthesis showed strong chlorosis, limited growth, and a strongly induced glycogen production in nitrogen-limited conditions, while those unable to synthesize glycogen did not (van der Woude, Angermayr, Veetil, Osnato, & Hellingwerf, 2014).

The capability to synthesize these polymers can affect complex interactions, such as plant—bacteria symbiosis. Many studies have proposed that PHB and glycogen are necessary to establish efficient symbiotic nitrogen fixation and plant growth promotion (Aneja et al., 2005; Wang et al., 2007). Analysis of the effect of PHB and glycogen in nodules formed by *Rhizobium leguminosarum* in leguminous plants showed opposite effects in the amount of starch accumulated by the plants around the nodules. This work reported that nodules formed by mutants that cannot synthesize PHB had very little starch, suggesting that carbon needed for bacteroid differentiation was provided by the plant. In the case of the glycogen synthesis-deficient mutants, large amounts of starch were observed, suggesting that bacteroids unable to synthesize this polymer require less carbon from the plant (Lodwig et al., 2005). It can thus be concluded that PHB acts as a carbon source for bacteroid differentiation, but the role of glycogen in this process still remains to be elucidated.

Although the relationship between PHAs and glycogen in isolated bacteria has very seldom been addressed, the dynamics of the metabolism of these polymers is very well known in mixed bacterial systems such as enhanced biological phosphorus removal (EBPR). EBPR is based on a series of metabolic processes, involving the formation of the inorganic polymer polyphosphate in mixed cultures in which nutrient availability and aeration conditions are alternated. Under anaerobic, nutrient-rich (feast) conditions, the organisms take up volatile fatty acids, and store the carbon as PHAs, using polyphosphate as energy source and glycogen degradation to obtain reducing power. Under aerobic, nutrient-limited (famine) conditions, PHAs are degraded and used as a carbon and energy source for growth, glycogen synthesis, and phosphate uptake (Oehmen et al., 2007) (Figure 2). The capability to carry out these reactions has been identified in *Candidatus* Accumulibacter phosphatis (Martin et al., 2006) and

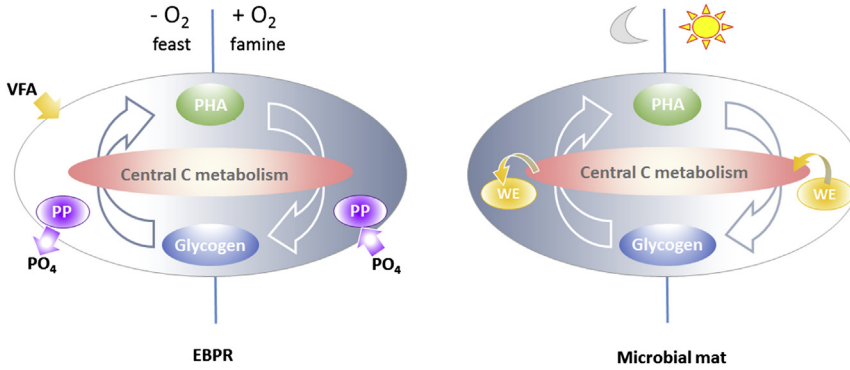

Figure 2 Cyclic relationship between polyhydroxyalkanoates (PHA) and glycogen in enhanced biological phosphorus removal (EBPR) and microbial mat anoxygenic phototrophic microorganisms. VFA, volatile fatty acids; WE, wax esters; and PP, polyphosphate. See text for further details.

Tetrasphaera sp. (Kristiansen et al., 2013), that are proposed to be among the main microorganisms responsible for phosphate removal in EBPR. Other organisms, such as *Candidatus* Competibacter denitrificans and *Candidatus* Contendobacter odensis, are also able to grow well in the system, but do not store polyphosphate, making their development undesirable in the reactors (McIlroy et al., 2014). The ability to accumulate and use PHAs and glycogen in a cyclic way has been observed in all these organisms, and the capacity to anaerobically store carbon as PHA for later use appears to be the principal selection pressure in EBPR.

The relationship between these two polymers has also been reported in natural systems subject to cyclic conditions. A recent metatranscriptomic study has reported that filamentous anoxygenic phototrophs in a thermophilic microbial mat community produce and utilize glycogen, PHAs, and wax esters, at different times during the diel cycle (Klatt et al., 2013). This work, focused on bacteria belonging to the Phylum Chloroflexi (*Chloroflexus* sp. and *Roseiflexus* sp.), proposes that PHAs and wax esters are synthesized at night, along with photosynthetic pigments, using carbon stored as glycogen, while in daytime, PHAs and wax esters are degraded and used as carbon and electron reserves to support photomixotrophy, and glycogen is accumulated (Klatt et al., 2013) (Figure 2).

These studies thus highlight the functional differences between these two common carbon storage compounds. These polymers are used by the cells to manage carbon resources in a variety of conditions, and they exhibit a key role in several ecosystems and in bacterial interactions.

7. CONCLUDING REMARKS

PHAs were originally described as reserve compounds used by bacteria as carbon and energy sources for different biochemical and cellular processes, but due to their thermoplastic properties, the majority of the studies focused on the biotechnological aspects of the polymer, and not on its relevance for the overall cell physiology. In spite of this, a large body of evidence is increasingly highlighting the important role that PHAs play in bacterial fitness and survival, and shows that polymer synthesis and degradation are integrated into bacterial metabolism. This metabolic feature, in turn, constitutes a valuable resource to increase metabolic versatility and flexibility in bacteria, with profound ecological implications that are only just starting to be revealed.

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