Genome-Wide Metabolic Reconstruction of the Synthesis of Polyhydroxyalkanoates from Sugars and Fatty Acids by *Burkholderia* Sensu Lato Species

Natalia Alvarez-Santullano ¹,³, Pamela Villegas ²,⁴, Mario Sepúlveda Mardones ¹,⁴, Roberto E. Durán ¹, Raúl Donoso ²,³, Angela González ¹, Claudia Sanhueza ⁴,⁵, Rodrigo Navia ⁴,⁶, Francisca Acevedo ⁴,⁵, Danilo Pérez-Pantoja ²,⁵ and Michael Seeger ¹,⁎

1. Introduction

Global plastic production reached 359 million tons in 2018, wherein 67.5% of the plastics are non-recycled, entering and polluting ecosystems [1,2]. Biodegradable bioplastics research and development have been focused towards replacing the fossil fuel-based...
plastics. Polyhydroxyalkanoates (PHAs) are attractive biopolymers due to their physico-chemical properties, their sustainable life cycle and their wide range of applications [3–7]. Bacteria may accumulate PHAs as intracellular granules, especially under high carbon availability, and nitrogen, phosphorus or oxygen limitation [8–10]. The PHAs are classified into short chain-length PHA (PHA\textsubscript{scl}), with monomers with from 3 to 5 carbon chain lengths, and medium chain-length PHA (PHA\textsubscript{mcl}), with monomers from 6 to 14 carbons. PHA\textsubscript{scl} are crystalline, stiff and brittle, whereas PHA\textsubscript{mcl} are flexible and possess lower crystallinity and tensile strength [11–13]. Industrial production of PHAs is still limited due to the elevated price (3.5 USD/kg), which is 3-fold more expensive than conventional plastics such as polypropylene (1.2–1.3 USD/kg) [14]. This limitation has been addressed through the search of novel bacterial strains with increased PHA productivity, low-cost substrates, and bioprocess optimization [3,15].

PHA\textsubscript{scl} and PHA\textsubscript{mcl} polymers are synthesized from precursors that are produced from: (i) sugars through metabolic pathways such as Entner–Doudoroff (ED), pentose phosphate (PP) and Embden–Meyerhof–Parnas (EMP) pathways, and (ii) fatty acids through β-oxidation or de novo synthesis [16–18]. The PHA\textsubscript{scl} polyhydroxybutyrate (PHB) is synthesized by the condensation of two molecules of acetyl-CoA by 3-ketothiolase PhaA into acetoacetyl-CoA, with a subsequent reduction by NADPH dependent acetoacetyl-CoA reductase PhaB into (R)-3-hydroxybutyryl-CoA (R-3HB-CoA). Finally, the PHA synthase PhaC polymerizes R-3HB-CoA into PHB [12,19]. The PHA synthases are classified in four classes. Class I, class III, and class IV PHA synthases are involved mainly in PHA\textsubscript{scl} synthesis, whereas class II PHA synthases polymerize PHA\textsubscript{mcl} [20]. Bacteria belonging to more than 90 genera produce PHAs [12]. Therefore, the search of bacterial metabolic networks and the genes involved in the PHA synthesis is a challenge [21–23].

Strains belonging to the 	extit{Burkholderia} sensu lato (s.l.) genera have been studied for their PHAs production capabilities. This group is referred as the species previously classified within the 	extit{Burkholderia} genus, and currently recognized as seven distinctive genera. The 	extit{Burkholderia} s.l. clade was divided in 2014 into the genus 	extit{Paraburkholderia}, which includes principally environmental strains, and the emended 	extit{Burkholderia} sensu stricto genus, which comprises mainly clinical and phytopathogenic strains [24]. Further phylogenetic analyses proposed a new genus, 	extit{Caballeronia}, whose species grouped as the outlined clade IIa of the 	extit{Paraburkholderia} genus [25]. Other species identified as outliers within the 	extit{Burkholderia} and 	extit{Paraburkholderia} genera have been also reclassified into 	extit{Trinickia}, 	extit{Mycetohabitans} and 	extit{Robbsia} genera [26]. Recently, another taxon has been described within 	extit{Burkholderia} s.l., the genus 	extit{Pararobbsia} [27]. Bacteria of the 	extit{Burkholderia} s.l. group possess generally large genomes (>6 Mbp) ranging from 3.7 to 11.5 Mbp and a G+C content between 58.5% to 68.5 mol%. Notably, strains belonging to these genera are adapted to a wide range of adverse environments and are metabolically versatile [28–31]. 	extit{Burkholderia} s.l. strains have been applied in bioremediation of pollutants, biocontrol of plant pathogens, plant-growth promotion, and synthesis of PHAs, enzymes, and siderophores [28,32–40]. The PHA synthesis by 	extit{Burkholderia} s.l. strains including 	extit{Paraburkholderia sacchari} LMG 19450\textsuperscript{T}, 	extit{Paraburkholderia xenovorans} LB400\textsuperscript{T}, 	extit{Burkholderia cepacia} ATCC 17759, 	extit{Burkholderia thailandensis} E264\textsuperscript{T}, 	extit{Trinickia caryophylli} DSM 50341\textsuperscript{T} and 	extit{Trinickia caryophylli} AS 1.2741 has been described, suggesting an intrinsic capability within this group to produce these biodegradable polymers [5,8,39–43]. 	extit{Paraburkholderia}, 	extit{Burkholderia} and 	extit{Trinickia} strains use a wide range of substrates, including carbohydrates and fatty acids, to generally synthesize PHA\textsubscript{scl} but also PHA\textsubscript{scl-mcl} copolymers [44,45]. The aims of this review are an extensive genomic-wide reconstruction of the metabolic pathways involved in the conversion of sugars and fatty acids into the synthesis of PHAs by 	extit{Burkholderia} s.l. representative and type strains, along with a genomic-based characterization of their PHA synthases and the organization of the \textit{pha} genes.
2. Synthesis of PHAs by *Burkholderia* Sensu Lato Strains

A literature search was carried out to identify *Paraburkholderia*, *Burkholderia*, *Caballero-nia*, *Trinickia*, *Mycetohabitans* and *Robbsia* strains applied for PHA synthesis. Data was gathered through the search in Web of Science (Clarivate Analytics, Philadelphia, PA, USA) using keywords such as “polyhydroxyalkanoate” “polyhydroxybutyrate”, “poly(3-hydroxybutyrate)” along with “Burkholderia”, “Paraburkholderia”, “Caballeronia”, “Trinickia”, “Mycetohabitans”, “Robbsia” with the addition of other synonyms from the period 1978–2019 (data retrieved on 9 December 2019).

The most reported PHA synthesized by *Paraburkholderia*, *Burkholderia* and *Trinickia* strains is PHB. The production of PHAs by *Caballeronia*, *Mycetohabitans* and *Robbsia* strains has not been reported. *P. sacchari* LMG 19450 T, *B. cepacia* ATCC 17759 and *B. thailandensis* E264 T showed to be relevant strains in PHA production (Table 1). However, PHA production has been also characterized in *P. xenovorans* LB400 T, *B. cepacia* IPT 048, *Burkholderia* sp. F24, *Burkholderia* sp. AIU M5M02, *B. contaminans* IPT 553 and *T. caryophylli* strains DSM 50341 T and AS 1.2741.

2.1. PHB Homopolymer Synthesis by Burkholderia Sensu Lato

PHB accumulation is promoted under nutrient limitation and high levels of carbon sources [15]. PHB production in *Paraburkholderia*, *Burkholderia* and *Trinickia* has been studied mainly under nitrogen limitation (Table 1). Interestingly, under phosphorus limitation higher PHB synthesis by *P. sacchari* LMG 19450 T was observed than under nitrogen limitation [8]. *B. thailandensis* E264 T, which was isolated from a rice soil in Central Thailand, is capable to synthesize PHA under nutrient balanced conditions [43].

Sugars are the most used carbon sources for the synthesis of PHB, although fatty acids have also been reported (Table 1). *B. thailandensis* E264 T synthesizes PHB from used cooking oil (UCO), while strain LMG 19450 T also produces PHB in presence of unsaturated fatty acid as co-substrates [44,44]. Interestingly, *T. caryophylli* DSM 50341 T produces PHB using gluconate or octanoate as the sole carbon source [46]. Glucose, gluconate, xylose, arabinose, mannitol, sucrose and fructose have been used for PHA production (Table 1). *P. sacchari* LMG 19450 T grown in glucose, sucrose and arabinose exhibited similar PHA production (4.0–4.2 g/L), while xylose reached a lower value (2.8 g/L). Conversely, *B. cepacia* ATCC 17759 displayed similar PHA production using glucose, fructose, sucrose (1.5–2.1 g/L) and xylose (1.5 g/L). The highest PHA yields were observed by *B. thailandensis* E264 T from fatty acids of used cooking oil (0.35) and by *P. sacchari* LMG 19450 T from sucrose (0.29), glucose (0.25–0.29) and arabinose (0.24). The differences in PHA production and PHA yield could be partly attributed to genetic determinants that are analyzed in this review.

Table 1. PHA homopolymers and copolymers synthesized by *Paraburkholderia* and *Burkholderia* strains.

| Strain          | Substrate | CDW (g/L) | PHA Type | PHA Concentration (g/L) | Limitation | Y_PHAS (g/g) | Reference          |
|-----------------|-----------|-----------|----------|-------------------------|------------|--------------|--------------------|
| Paraburkholderia sacchari LMG 19450 T (IPT 101, DSM 17165, LFM 101, CCT 6971) | Glu       | 5.0–6.4   | PHB      | 0.35–4.0                | Nitrogen   | 0.25–0.29    | [14,41,47]         |
|                 | Xyl       | 2.9–6.3   | PHB      | 0.49–2.8                | Nitrogen   | 0.05–0.26    | [8,41,47,48]       |
|                 | Ara       | 7.4       | PHB      | 0.5–4.7                 | Nitrogen   | 0.24         | [14,47]           |
|                 | Man       | 6.9       | PHB      | 4.2                     | Nitrogen   | 0.21         | [49]               |
|                 | Gal       | 4.9       | PHB      | 2.2                     | Nitrogen   | 0.11         | [49]               |
|                 | Scr       | 6.14      | PHB      | 4.2                     | Nitrogen   | 0.29         | [50]               |
|                 | Glu/Fatty acids | 1.25–2.4 | P(3HB-co-3HV) | 0.4–0.9              | -          | -            | [44]               |
|                 | Glu/GBL, 4HBA | 1.8–6.6 | P(3HB-co-4HB) | 0.4–3.1              | Nitrogen   | 0.01–0.1 ** | [44,51,52]        |
|                 | Glu/HxA   | 2.1       | P(3HB-co-3HHx) | 1.1                  | Nitrogen   | -            | [44]               |
| Paraburkholderia xenovorans LB400 T | Glu       | -         | PHB      | (40% w/w)               | Nitrogen   | -            | [40,53]  |
|                 | Xyl       | -         | PHB      | NR                      | Nitrogen   | -            | [54]               |
|                 | Man       | -         | PHB      | NR                      | Nitrogen   | -            | [54]               |
2.2. PHA Copolymer Synthesis by Burkholderia Sensu Lato

The PHA copolymers synthesized by Paraburkholderia, Burkholderia and Trinickia strains are composed of PHA<sub>scl</sub> and PHA<sub>mcl</sub> monomers. Different metabolic pathways can supply the intermediates for PHA copolymers. <i>P. sacchari</i> LMG 19450<sup>T</sup> produces the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)) from glucose and odd-chain fatty acids as co-substrates, increasing 3-hydroxyvaleryl (3HV) content when valeric acid is supplied as co-substrate [44]. The 3HV content decreases with longer odd-chain fatty acids supplied, due to the higher level of acetyl-CoA generated through β-oxidation, which increased 3HB monomer synthesis. However, when even-numbered saturated or unsaturated fatty acids are supplied as co-substrates, strain LMG 19450<sup>T</sup> produces only PHB [44].

The synthesis of P(3HB-co-3HV) by <i>Paraburkholderia</i> and <i>Burkholderia</i> strains fed with glucose, succinate, xylose and glycerol in presence of a co-substrate such as levulinic acid (LA), valeric acid and propionic acid has been reported (Table 1). LA may be obtained by acid catalysis of low-cost renewable sources, including cellulose-forest and agricultural residues, therefore, is an interesting co-substrate for PHA production in bacteria resistant to the LA-related toxicity [60,61]. Strains <i>P. sacchari</i> LMG 19450<sup>T</sup> and <i>B. contaminans</i> IPT 553 produce the PHA<sub>scl-mcl</sub> co-polymer poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (P(3HB-co-3HHx)), poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), P(3HB-co-3HHx), poly(3-hydroxybutyrate-co-3-hydroxyoctanoate), (P(3HHx-co-3HO-co-3HD)), poly(3-hydroxyoctanoate-co-3-hydroxydecanoate), UCO: used cooking oil. CDW: cell dry weight; NR: not reported; NL: not limited; * no growth reported, ** g P4HB/g GBL.

### Table 1. Cont.

| Strain                | Substrate | CDW (g/L) | PHA Type | PHA Concentration (g/L) | Limitation | Y<sub>P<sub>EA</sub>s</sub> (g/g) | Reference |
|-----------------------|-----------|-----------|----------|-------------------------|------------|-------------------------------|-----------|
| Burkholderia cepacia  | Glu       | 2.6       | PHB      | 1.5                     | Nitrogen   | -                             | [55]      |
| ATCC 17759 (DSM 50181)| Fru       | 5         | PHB      | 2                       | Nitrogen   | 0.07–0.174                   | [55,56]   |
|                       | Xyl       | 2.6       | PHB      | 1.5                     | Nitrogen   | 0.11                          | [55]      |
|                       | Scr       | 4.2       | PHB      | 2.1                     | Nitrogen   | 0.18                          | [50]      |
|                       | Xyl/LaA   | 3.3       | P(3HB-co-3HV) | 2.4                 | -          | -                             | [57]      |
|                       | Glu/PA    | 1.6–1.8   | P(3HB-co-3HV) | 0.2–1.0               | -          | -                             | [58]      |
| Burkholderia thailandensis E264 | UCO (fatty acids) | 12.6 | PHB | 7.5 | NL | 0.35 | [43] |
| Burkholderia contaminans Kad1 | Waste glycerol/VA | 5.6 | P(3HB-co-3HV) | 1.96 | - | - | [58] |
| Burkholderia contaminans IPT 553 | Glu/Scr | 2.3–4.9 | P(3HB-co-3HDd) | 0.85–1.176 | - | - | [45] |
| Trinickia caryophylli AS 1.2741 | OA | 1.084 | P(3HHx-co-3HO-co-3HD) | 0.26 | - | - | [59] |
|                       | OA        | 1.159     | P(3HHx-co-3HO-co-3HD) | 0.23 | - | - | - |

Glu, glucose; Xyl, xylose; Ara, arabinose; Man, mannitol; Gal, galactose; Scr, sucrose; Gnt: gluconate; Fatty acids: propionic; valeric (VA), heptanoic, nonanoic, undecanoic acid; 3HB, 3-hydroxybutyryl; 3HV: 3-hydroxyvaleryl; 3HDd: 3-hydroxydodecanoyl; GBL: gamma-butyrolactone; 4HBA: 4-hydroxybutyric acid; HxA: hexanoic acid; Fru, fructose; NaA: Lauric acid; PA, propionic acid; OA, octanoic acid; P(3HB-co-3HV), poly(3-hydroxybutyrate-co-3-hydroxyvalerate); P(3HB-co-4HB), poly(3-hydroxybutyrate-co-4-hydroxybutyrate); P(3HB-co-3HHx), poly(3-hydroxybutyrate-co-3-hydroxyhexanoate); P(3HHx-co-3HO-co-3HD), poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate-co-3-hydroxydecanoate); UCO: used cooking oil. CDW: cell dry weight; NR: not reported; NL: not limited; * no growth reported, ** g P4HB/g GBL.
of 37 g/L with 5% 4HB monomer [51]. 4-Hydroxybutanoate is partially converted into 4-hydroxybutyryl-CoA but is mainly oxidized into succinic acid semialdehyde and succinic acid, which is further converted to acetyl-CoA and then into 3-hydroxybutyryl-CoA [51]. The PHA precursors provided by diverse catabolic pathways of different substrates have been related to PHA productivity and monomeric composition [21,63–65]. However, the diversity of metabolic pathways and genetic determinants related to PHA synthesis in Burkholderia s.l. bacteria have only partly been reported [66].

3. Metabolism of Sugars and Fatty Acids in Burkholderia Sensu Lato

At March 2021, more than 150 validly published species represent the Burkholderia s.l. group, including: 78 Paraburkholderia spp., 34 Burkholderia spp., 27 Caballeronia spp., 7 Trinickia spp., 2 Myceotohabitans spp., 1 Robbsia spp., and 2 Pararobbsia spp. [67]. Based on the proportion of species described in Burkholderia s.l., and identifying representative clades within each genus, a genome selection was carried out for further analysis. Selection was performed using AnnoTree [68], the web browser based on taxonomy information derived from the Genome Taxonomy Database phylogeny [69]. Burkholderia s.l. species were further selected based on their phylogenomic placement at the infragenic level, the availability of a metabolic network in curated databases [70] or a genome sequence in public databases. The metabolism of 37 selected strains of Burkholderia s.l., each belonging to a different species, was analyzed to identify genetic determinants and pathways involved in the conversion of sugars, fatty acids and related compounds for the production of PHAs. The selection consisted of 13 Burkholderia, 14 Paraburkholderia, 6 Caballeronia, 2 Trinickia, 1 Myceotohabitans and 1 Robbsia genomes (Table 2). Pararobbsia strains were not analyzed in this review as both species comprised in the genus, Pararobbsia alpina and Pararobbsia silviterrae, are newly proposed species with no relevant information besides their species description.

| Strain                      | Accession Number | Chr | Plasmids | Size (Mbp) | Contigs | CDS  | G+C Content (mol%) |
|-----------------------------|------------------|-----|----------|------------|---------|------|-------------------|
| Burkholderia cepacia ATCC 25416T | GCA_006094315    | 3   | 2        | 8574       | 5       | 7619 | 66.59             |
| Burkholderia contaminans MS14 | GCA_001029145    | 3   | 0        | 8509       | 3       | 7494 | 66.38             |
| Burkholderia cenocepacia J2315 | GCA_902830575    | -   | 0        | 7911       | 89      | 7105 | 66.99             |
| Burkholderia stabilis ATCC BAA-67T | GCA_01742165     | 2   | 0        | 8528       | 3       | 7352 | 66.42             |
| Burkholderia pyrrocinia DSM 10685T | GCA_001028665    | 3   | 1        | 7961       | 4       | 6920 | 66.46             |
| Burkholderia vietnamiensis LMG 10929T | GCA_902830295   | -   | 0        | 6876       | 65      | 5397 | 66.89             |
| Burkholderia ambifaria AMMDT | GCA_000959545    | 3   | 1        | 7528       | 4       | 6548 | 66.77             |
| Burkholderia stagnalis LMG 28156T | GCA_902830275    | -   | 0        | 8032       | 149     | 7039 | 67.23             |
| Burkholderia multivorans ATCC 17616 | GCA_000010545    | 3   | 1        | 7009       | 4       | 6262 | 66.69             |
| Burkholderia thailandensis E264T | GCA_000012365    | 2   | 0        | 6724       | 2       | 5656 | 67.73             |
| Burkholderia mallei ATCC 23344T | GCA_000011705    | 2   | 0        | 5836       | 2       | 4820 | 68.49             |
| Burkholderia glumae DSM 2196T | GCA_902832765    | -   | -        | 6662       | 142     | 5623 | 68.34             |
| Burkholderia phymatum STM 815T | GCA_000020045    | 2   | 2        | 8676       | 4       | 7898 | 62.75             |
| Paraburkholderia graminis PHS1 | GCA_003307085    | 2   | 1        | 7508       | 3       | 6510 | 62.83             |
| Paraburkholderia caledonica PHRS4 | GCA_003307045    | 2   | 1        | 7305       | 3       | 6042 | 61.93             |
| Paraburkholderia aromaticivorans BN5T | GCA_002278075    | 2   | 6        | 8908       | 8       | 7753 | 62.94             |
| Paraburkholderia xenovorans LB400T | GCA_000756045    | 2   | 1        | 9703       | 3       | 8321 | 62.63             |
| Paraburkholderia phytofirmans PsJN | GCA_000201255    | 2   | 1        | 8215       | 3       | 7175 | 62.29             |
| Paraburkholderia fungorum ATCC BAA-463T | GCA_003386235    | 3   | 1        | 9039       | 4       | 7898 | 61.75             |
| Paraburkholderia caffinilgica CFI | GCA_003386235    | 2   | 1        | 8324       | 3       | 7142 | 62.21             |
| Paraburkholderia sprettiae WSM5005T | GCA_001165575    | 2   | 3        | 7829       | 5       | 6699 | 63.21             |
| Paraburkholderia megalopolitana DSM 23650T | GCA_900113825    | -   | -        | 7607       | 32      | 6571 | 62.07             |
| Paraburkholderia terrae DSM 17804T | GCA_002902925    | 4   | 0        | 10,062     | 4       | 8754 | 61.92             |
| Paraburkholderia hospita DSM 17164T | GCA_002902965    | 5   | 1        | 11,328     | 6       | 9975 | 61.79             |
| Paraburkholderia phytofirmans STM 815T | GCA_000200455    | 2   | 2        | 8676       | 4       | 7405 | 62.29             |
| Paraburkholderia sacchari DSM19450T | GCA_000786155    | 3   | 1        | 7358       | 21      | 6341 | 64.01             |
| Paraburkholderia tropica DSM 22274T | GCA_902833865    | -   | -        | 6874       | 72      | 6002 | 60.15             |

Table 2. Genome characteristics of 37 Burkholderia sensu lato type and representative strains.
Table 2. Cont.

| Strain                    | Accession Number | Chr | Plasmids | Size (Mbp) | Contigs | CDS | G+C Content (mol%) |
|---------------------------|------------------|-----|----------|------------|---------|-----|-------------------|
| *Caballeronia udeis* LMG 27134<sup>T</sup> | GCA_001544555 * | -   | -        | 10,052     | 242     | 8774 | 60.04             |
| *Caballeronia gluthei* LMG 14190<sup>T</sup> | GCA_902833485 | -   | -        | 8637       | 356     | 7660 | 64.41             |
| *Caballeronia insecticola* RPE64<sup>T</sup> | GCA_000402035 | 3   | 2        | 6964       | 5       | 6266 | 63.15             |
| *Caballeronia cordobensis* LMG 27620<sup>T</sup> | GCA_001544575 * | -   | -        | 8208       | 74      | 7428 | 63.69             |
| *Caballeronia grimmiae* LMG 27580<sup>T</sup> | GCA_000698555 | -   | -        | 6704       | 160     | 6024 | 63.02             |
| *Trinickia caryophylli* DSM 50341<sup>T</sup> | GCA_002879875 | -   | -        | 6581       | 158     | 5626 | 64.62             |
| *Trinickia symbiotica* JPY-345<sup>T</sup> | GCA_00198775 | -   | -        | 3750       | 3       | 2875 | 60.71             |
| *Mycetohabitans rhizoxinica* HKI 454<sup>T</sup> | GCA_001544575<sup>*</sup> | -   | -        | 8208       | 74      | 7428 | 63.69             |
| *Robbsia andropogonis* LMG 2129<sup>T</sup> | GCA_002833845 | -   | -        | 633        | 77      | 5183 | 58.86             |

Chr, number of chromosomes; CDS, coding sequences; type strains are marked with a T superscript; (*) a second version of the genome.

The evolutionary relatedness between each taxa against the genetic and metabolic traits involved in carbohydrates and fatty acids assimilation was assessed. For this purpose, a phylogenomic analysis of the 37 selected strains was conducted. A phylogeny of 38 concatenated core genes was constructed using the Phylophlan software [71], followed by a maximum-likelihood analysis. The bootstrap confidence values were calculated with 1000 replicates, while values below 50% were not shown (Figure 1A). As expected, six clades were clearly distinguished, each of them representing one genus belonging to *Burkholderia* s.l. (*Burkholderia* sensu stricto, red branch; *Paraburkholderia* green branch; *Caballeronia*, purple branch; *Trinickia*, yellow branch; *Mycetohabitans*, dark blue branch; and *Robbsia*, pink branch; Figure 1A). To further corroborate each species placement, an average nucleotide identity based on Mummer (ANIm) analysis was conducted. Genomic index values supported the clades identified by the Phylophlan software (Figure 1B). Among the 37 *Burkholderia* s.l. genomes analyzed, ANIm values were below the current cut-off for species delineation (>95–96%, Richter and Roselló-Mora, 2009), excluding *Paraburkholderia hospita* DSM 17164<sup>T</sup> and *Paraburkholderia terrae* DSM 17804<sup>T</sup> (ANIm 95.4%, blue square, Figure 1B). The taxonomic classification of the *Paraburkholderia* subgroup was assessed by Pratama et al., obtaining the same ANIm value (95.42%) when comparing *P. terrae* and *P. hospita* type strains. The authors proposed a larger species “cluster”, represented by *P. hospita* and supported by phylogeny based on 16S rRNA gene sequence, multilocus sequence analysis using 7 concatenated genes, ANIm, tetrancleotide frequencies (TETRA), and comparative genomics [72].

For the identification of genetic determinants involved in the metabolism of sugars and fatty acids, genome-based metabolic networks were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. For the strains without a prior network (e.g., *Caballeronia* spp., *T. caryophylli* DSM 50341<sup>T</sup>, *Trinickia symbiotica* JPI-345<sup>T</sup>, *Robbsia andropogonis* LMG 2129<sup>T</sup>) a genome-based reconstruction was performed manually by a Bidirectional Best Hit (BBH) approach. Absent enzymes from the metabolic networks (e.g., PhaG, PhaJ, GlK, GlpD, BktB) were manually searched through a BBH approach, against amino acid sequences with experimental evidence obtained from the Swiss-Prot database, using a threshold of ≥30% identity and ≥70% coverage. For the identification of each genetic determinant, the genomic context was analyzed, and all reactions reported in the present study were manually curated and depicted in Figures 2 and 3.
In general, *Paraburkholderia* and *Caballeronia* strains show higher gene redundancy than the rest of the *Burkholderia* s.l. genera in the EMP, PP of carbohydrates metabolism (Figure 4), and β-oxidation and fatty acid de novo synthesis pathways of fatty acids metabolism (Figure 5). For example, in carbohydrate metabolism (Figure 4) *Paraburkholderia* and *Caballeronia* strains have 1 to 3 genes coding for glucokinase (*glk*), glucose-6-phosphate dehydrogenase (*zwf*), and transketolase (*tkt*). On the other hand, specific clades of the *Burkholderia* genus (*Burkholderia cepacia* complex) presented two copies of fructokinase *scrK* gene. Similarly, in *Paraburkholderia* and *Caballeronia* genera, more copies of the β-oxidation *fadE* and *fadA* genes along with fatty acid de novo synthesis *fabG* and *fabI* genes are present (Figure 5). Interestingly, the essential enzyme for unsaturated fatty acid synthesis in *E. coli*, class I ketoacyl-ACP synthase (KAS) that is encoded by the *fabB* gene, showed higher presence in *Paraburkholderia* and *Caballeronia* strains than the rest of *Burkholderia* s.l. genera [73]. On the other hand, genes related to PHA metabolism are highly variable among species, especially in the *Paraburkholderia* and *Caballeronia* genera, probably due to horizontal gene transfer events described previously [74]. In addition, five *Paraburkholderia*, two *Caballeronia* and the *Burkholderia* cepacia complex strains possess additional copies of the *phaA* and *phaB* genes encoded in two gene clusters harboring a phasin gene, the *phaPBA* cluster and *phaPB* gene cluster. Finally, *Mycetohabitans rhizorzinica* HKI 454 T possess different genetic profile due to the absence of fructose, xylose, sucrose, mannitol and arabinose assimilation pathways along with a low copy number in EMP, PP, β-oxidation and fatty acid de novo synthesis pathways. The substrate assimilation by *Burkholderia* s.l. strains was reviewed in Table S1 [75–95]. Remarkably *Mycetohabitans rhizorzinica* HKI 454 T is the only strain unable to grow on glucose as the sole carbon source.

**3.1. Metabolism of Sugars for PHA Production in Burkholderia Sensu Lato**

Sucrose, glucose, fructose, xylose, mannitol, gluconate and glycerol were the analyzed substrates for the genome-based reconstruction of metabolic pathways in *Burkholderia* s.l.
strains for their conversion into PHA, as they are the main compounds reported for PHA production by these bacteria (Table 1).

In Burkholderia sensu stricto strains, sucrose is hydrolyzed by sucrose hydrolase $\beta$-fructofuranosidase (SacB) into glucose and fructose (Figure 2). In contrast, most of Paraburkholderia and Caballeronia strains are unable to metabolize sucrose, except for Paraburkholderia graminis PHS1 and Paraburkholderia megapolitana LMG 23650T (Table S1), which probably break down sucrose through $\alpha$-glucosidase (MalL). T. caryophylli DSM 50341T is able to import sucrose through a phosphoenolpyruvate transferase system (PTS) family transporter, yielding sucrose-6-phosphate that is subsequently transformed to glucose-6-phosphate (G6P) and D-fructose by a sucrose-6-phosphate hydrolase. Except for Mycetohabitans rhizoxinica HKI 454T, mannitol is oxidized by mannitol dehydrogenase (MltK) or arabinitol 4-dehydrogenase (DaiD) into fructose, yielding NADH equivalents. All the strains have ABC transporter genes for glucose import. Glucose is subsequently phosphorylated into G6P by glucokinase enzyme, encoded by the glk gene that is present in all analyzed strains. Particularly, Paraburkholderia and Caballeronia genomes possess an additional polyphosphate-dependent glucokinase gene (polyP-Glk). The closest relative of these enzymes is the polyP-Glk of Anabaena sp. PCC 7120, described to participate in nitrogen deprivation stress resistance in this cyanobacterium [96]. PolyP-Glk is also present in the phytopathogens Burkholderia plantarii ATCC 43733T and Burkholderia glumae LMG 2196T (Figure 4). Additionally, in Caballeronia strains, excluding Caballeronia sordidicola LMG 22029 and Caballeronia udeis LMG 27134T glucose may enter through a phosphotransferase system (PTS) family transporter, producing G6P (Figures 2 and 4) [97,98]. Most of the Burkholderia s.l. strains probably incorporate fructose by an ABC family transporter, and then fructose is phosphorylated by fructokinase into fructose-6-phosphate (F6P) (Figures 2 and 4). In contrast, in P. graminis PHS1 and B. thailandensis E264T, fructose may be transported into the cell through a PTS system that phosphorylates fructose into fructose-1-phosphate (F1P) [99]. F6P and F1P can be phosphorylated by a 6-phosphofructokinase (Pfk, pfk gene) into fructose-1,6-bisphosphate (F1,6bP) and then metabolized through the glycolytic EMP pathway (Figure 2; [99]) Alternatively, F1,6bP may enter the gluconeogenic EMP pathway to generate F6P that is converted by a G6P isomerase into G6P [99]. Interestingly, from all the analyzed strains, only 53% Burkholderia, 28% Paraburkholderia and both Trinickia genomes possessed a complete EMP pathway (Figures 2 and 4). In contrast, the EMP pathway is incomplete in the rest of the analyzed strains, due to the absence of the pfk gene. Notably, in all the strains with complete EMP pathway, the pfk gene is located from 0 to 12 ORFs upstream of a phaC gene organization. The interrupted EMP pathway due to pfk absence has also been reported in the Ralstonia genus, marine bacteria of Alphaproteobacteria, Gammaproteobacteria and Flavobacteria classes, and Pseudomonas strains [99–104].
Figure 2. Proposed metabolic pathways from sugars and glycerol into the synthesis of polyhydroxybutyrate of Burkholderia sensu lato genera. Each reaction is represented with an arrow with the respective enzyme depicted with a number. Compounds with their name underlined are carbon and energy sources for these bacteria. Enzymes present in all analyzed genomes are marked with “⌘”. 1, β-fructofuranidase (FFase), α-glucosidase (MalL); 2, mannitol dehydrogenase (MDH); 3, glucokinase (GLK); 4, glucose phosphoenolpyruvate transferase system (PTS) family transporter (glu-EII); 5, fructokinase (FK); 6, glucose-6-phosphate (G6P) isomerase (G6PI); 7, 6-phosphofructokinase (6PFK); 8, fructose-1,6-phosphatase (FBPase); 9, fructose-1,6-biphosphate aldolase (FBA); 10 triosephosphate isomerase (TIM); 11, Fructose PTS family transporter (fru-EII); 12, 1-phosphofructokinase (1PFK); 13, G6P dehydrogenase (G6PDH); 14, 6-phosphogluconolactonase (6PGnL); 15, phosphogluconate dehydrogenase (6PGntD); 16, ribulose-5-phosphate epimerase (R5PE); 17, ribulose-5-phosphate isomerase (R5PI); 18, transketolase (TKT); 19, transaldolase (TAL); 20, gluconate kinase (GntK); 21, phosphogluconate dehydratase (PGntDT); 22, 2-keto-3-deoxyphosphogluconate (KDPnt) aldolase (KDPGA); 23, xylose isomerase (XI); 24, xylulokinase (XK); 25, glycerol kinase (GK); 26, glycerol-3-phosphate dehydrogenase (G3PDH); 27, L-arabinose-1-dehydrogenase (LADH); 28, arabinolactonase (AL); 29, arabinonate dehydratase (AD); 30, L-KDA dehydratase (LKDADT); 31, α-ketoglutarate semialdehyde dehydrogenase (KGSADH); 32, ketothiolase (PhaA); 33, acetoacetyl-CoA reductase (PhaB); 34, PHA synthase (PhaC). DHAP: dihydroxyacetone phosphate; α-KS: α-ketoglutarate semialdehyde; TCA; tricarboxylic acid cycle. Homology prediction of 27 strains was performed using the curated metabolic networks of the Kyoto Enzyme and Genomes Database (KEEG) and manual Blast search through Bidirectional Best Hit approach (BBH). For the 10 strains belonging to novel genera (e.g., *Caballeronia*, *Trinickia*, *Mycetohabitans*, *Robbsia*) a manual reconstruction through BBH was performed. An amino acid sequence identity of >30% and ≥70% coverage was used as threshold in function of the gene context for homology prediction. Genomes were retrieved from Refseq database.
In *Burkholderia* s.l. strains, G6P, obtained from sugar metabolism and gluconeogenic EMP, is oxidized in the pentose phosphate (PP) pathway by a G6P dehydrogenase (G6PDH), enzyme encoded by the *zwf* gene, yielding gluconolactone-6-phosphate (GL-6P) and NADPH. 6-Phosphogluconolactonase (6PGL) hydrolyzes GL-6P into gluconate-6-phosphate (gluconate-6P), which is channeled through the ED or PP pathways (Figure 2). Gluconate-6P can also be obtained from gluconate by gluconokinase in *Burkholderia* s.l. strains. ED and PP pathways were complete and conserved in the *Burkholderia* s.l. strains (Figure 4). G6PDH is a key enzyme in the oxidative branch of PP pathway during glucose oxidation [105], and in *Burkholderia* s.l. strains the *zwf* gene coding for this enzyme is located in the *zwf-pgl-glk* organization. *Burkholderia*, *Caballeronia*, *Mycetohabitans* and *Trinickia* strains have up to 2 *zwf* gene copies (Figure 4), while *Paraburkholderia* and *Robbsia* strains possess a third *zwf* gene copy located next to *polyP-glk* gene and a glycogen debranching gene. The *Burkholderia* phytopathogen subgroup also possesses the *polyP-glk-zwf* arrangement. This suggests that the *zwf* gene redundancy in these bacteria may play a role during the adaptation of these organisms in environments of variable nutrient availability, as mentioned before for *polyP-glk* gene and the NADPH synthesis via the oxidative branch of PP pathway [96,105]. During the conversion of G6P into pyruvate via the ED pathway 1 molecule of NADPH, 1 molecule of NADH and 2 ATP are produced, whereas only 1 molecule of NADH but 3 ATP are synthesized through the EMP pathway. In comparison, the degradation of G6P via the PP pathway produces up to 6 molecules of NADPH, 1 molecule of NADH and 2 ATP. In *Pseudomonas*, the carbon derived from gluconate-6P is funneled through ED pathway and then through the gluconeogenic EMP and oxidative PP pathways to yield NADPH [99,100]. Furthermore, metabolic flux analysis in *Pseudomonas putida* KT2440 and *Pseudomonas protegens* Pf-5 (both strains lack the *pfk* gene) demonstrated that ~90% of glucose enters the ED pathway through gluconate-6P, in part attributed to the lower protein expenses leading to NADPH accumulation [99,101,106]. It has also been observed that the deficiency in the EMP pathway in diverse bacteria of Alphaproteobacteria, Gammaproteobacteria and Flavobacteria classes leads to an increase of NADPH supplied by the ED pathway [102,103]. In *Pseudomonas* and *Chromohalobacter* genera, the oxidative phase of PP, ED and the gluconeogenic EMP pathway generate a cycle that promotes biosynthetic precursors and NADPH equivalents [99,101,106]. When the EMP pathway is completed by the insertion of the *pfk* gene in *P. putida* KT2440 strain, this cycle may be bypassed, decreasing ATP yield (~50%) and reducing NADPH/NADP+ ratio from 1.4 to ~0.6 [107]. In addition, *P. putida* KT2440 segregates the carbon provided from glucose and benzoate in the upper EMP-ED-PP cycle and tricarboxylic acid cycle (TCA cycle), respectively, in order to supply biosynthetic compounds flux and NADPH demands [100]. Similarly, *Ralstonia* species lack *pfk* and gluconate-6-phosphate dehydrogenase (*gnd*) genes, resulting in interrupted EMP and oxidative PP pathways, placing ED pathway as the main route for glucose oxidation and NADPH production, according to carbon isotope labeling studies [104]. NADPH provides reducing power to endure oxidative stress by reducing antioxidative molecules (e.g., glutathione, thioredoxin and alkyl hydroperoxides) and the synthesis of compounds related to stress resistance (e.g., ectoines, PHAs) [101,108,109]. Model bacteria for the degradation of aromatic compounds such as *P. xenovorans* LB400 and *P. putida* KT440 possess strong antioxidative systems that avoid the accumulation of reactive oxygen species (ROS) during degradation of aromatic compounds [110–112]. Therefore, maintaining a high NAD(P)H/NAD(P)+ ratio could contribute to the detoxification of ROS during aromatic degradation [111]. Sacomboio et al. [113] reached a 2-fold increase in PHA production related to a 2-fold increment in the NADPH/NADP+ ratio due the increased expression of G6PDH by mutating the *ntrC* regulator in the *Burkholderiales* bacterium, *Herbaspirillum seropedicae*. The metabolic traits described in *Pseudomonas* are probably also involved in the PHA metabolism in *Burkholderia* s.l. genera. These metabolic networks may allow the adaptation under the fluctuating environmental conditions where species of *Pseudomonas* and *Burkholderia* s.l. strains inhabit [107,111,114]. However, further
insights are needed to confirm a cyclic metabolic flux in EMP, ED and PP pathways that promotes PHA synthesis in Burkholderia s.l. bacteria.

Figure 3. Proposed metabolism of fatty acids associated to the synthesis of PHAs in Burkholderia sensu lato genera. Each reaction is represented with an arrow, with the respective enzyme or enzymes that catalyze the reaction depicted with a number. Enzymes present in all genomes are marked with “£”.

1, acetyl-CoA carboxylase (ACC); 2, malonyl-CoA-ACP transacylase (MAT); 3, class I-III β-ketoacyl-ACP synthases (KAS); 4, 3-ketoacyl-ACP reductase (KR); 5, β-hydroxyacyl-ACP-dehydratase (HAD); 6, enoyl-ACP reductase (ENR); 7, acyl-CoA dehydrogenase (ACAD); 8, multifunctional S-specific enoyl-CoA hydratase-hydroxyacyl-CoA dehydratase (HCDH/ECH); 9, β-ketoacyl-CoA thiolase (KAT); 10, 3-hydroxybutryl-CoA epimerase (HB3E); 11, R-specific enoyl-CoA hydratase (R-ECH); 12, PHA synthase (PhaC); 15, β-ketothiolase (PhaA); 16, β-ketothiolase (BktB); 17, acetoadetyl-CoA reductase (PhaB). P(3HB-co-3HA), poly(3-hydroxybutyrate-co-3-hydroxyacyl) represents any copolymer containing PHA_mcl monomers (e.g., 3-hydroxybutyrate) and PHA_mcl monomers (e.g., 3-hydroxyacyl).

Homology prediction of 27 strains was performed using the curated metabolic networks of the Kyoto Enzyme and Genomes Database (KEGG) and manual Blast search through Bidirectional Best Hit approach (BBH). For the 10 strains belonging to novel genera (e.g., Caballeronia, Trinickia, Mycetohabitans, Robbsia) a manual reconstruction through BBH was performed.

An amino acid sequence identity of >30% and ≥70% coverage was used as threshold in function of the gene context for homology prediction. Genomes were retrieved from Refseq database.

Xylose (“wood sugar”) and arabinose are present in agricultural by-products used for PHA production [8]. In Burkholderia s.l. strains, xylose is isomerized to xylulose and then phosphorylated into xylulose-5-phosphate (Xyl-5P) by xylulose isomerase and xylulokinase [115]. The xylose isomerase pathway is conserved in the Paraburkholderia and Burkholderia sensu stricto strains. Xyl-5P is funneled into the non-oxidative PP pathway. Xyl-5P and ribose-5-phosphate are converted by transketolase and transaldolase via sedoheptulose-7-phosphate, glyceraldehyde-3-phosphate (GlyA-3P) and erythrose-4-phosphate into F6P and GlyA-3P (Figure 2). F6P is isomerized in the gluconeogenic EMP pathway to G6P and channeled into ED pathway, yielding NADPH. PHB production using xylulose as the sole carbon source have been reported in P. sacchari LMG 19450^T, showing...
40% lower PHB production compared to glucose [41]. This difference has been related to the higher reductive power provided by glucose compared to xylose due to the additional NADPH production by G6PDH and GL-6P dehydrogenase during glucose catabolism via oxidative PP pathway than the xylose isomerase pathway. Conversely, B. cepacia ATCC 17759 showed similar PHB concentration from glucose and xylose (Table 1) [55]. This may be explained by an alternative xylose degradation pathway, described previously in Caulobacter crescentus, which exhibit NAD(P)H generation by xylose dehydrogenase (XyLA) and α-ketoglutaric semialdehyde dehydrogenase (XylC) [115]. However, this alternative catabolic pathway is incomplete since the xylA gene was not found in none of the analyzed strains and ATCC 17759 draft genome. Furthermore, B. glumae LMG 21967, B. plantarii ATCC 43733T and T. caryophylli DSM 50341T can assimilate xylose as the sole carbon source (Table S1) although both pathways are incomplete (Figure 4), suggesting the presence of an alternative xylose degradation route or that the enzymes of these pathways are distantly related. The xylose isomerase pathway is conserved in the Paraburkholderia genus, 9 Burkholderia species and 2 Caballeronia species (Figure 4).

PHA production from arabinose has been reported in P. sacchari LMG 19450T [14,47] and most of the analyzed strains assimilate arabinose (Table S1). The ara genes of the classical arabinose catabolic pathway [116] were not found in the analyzed strains (Figure 4). This suggests a second arabinose catabolic pathway, involving non-phosphorylated metabolic intermediates [116]. L-arabinose is converted by L-arabinose 1-dehydrogenase, L-arabinono-lactonase, and L-arabonate dehydratase to L-2-keto-3-deoxyarabonate (L-KDA) (Figure 2). L-KDA is transformed by L-KDA dehydratase into α-ketosemialdehyde (α-KS). Finally, α-KS is converted to α-ketoglutarate by the type I α-ketoglutaric semialdehyde dehydrogenase (KGSADH) enzyme encoded by the arabinose inducible araE gene [117]. This pathway is conserved in all Burkholderia sensu stricto strains except for Burkholderia mallei ATCC 23344T, B. thailandensis E264T and Burkholderia stabilis ATCC BAA-67T. Conversely, 10 Paraburkholderia, 4 Caballeronia, T. caryophylli DSM 50341T, M. rhizoxinica HKI 454T and R. andropogonis LMG 2129T strains have an incomplete arabinose degradation pathway. One group of eight Paraburkholderia, Caballeronia glathei LMG 14190T and M. rhizoxinica HKI 454T strains lacks the araA gene encoding L-arabinose dehydrogenase (ADH) or other arabinose degradation genes. Interestingly all these strains assimilate this substrate, except B. mallei ATCC 23344T (Table 1 and Table S1), suggesting an alternative arabinose assimilation pathway. On the other hand, a second group of 2 closely related Paraburkholderia, 4 Caballeronia strains and B. thailandensis E264T lacks the araE gene encoding KGSADH enzyme (Figure 4) although they can assimilate L-arabinose (Table S1). Interestingly, all these strains possess 1–5 additional aldH gene copies encoding type II and III KGSADH enzymes, which are induced in Azospirillum brasíense by D-glucarate/D-galactarate and hydroxyproline, respectively. These KGSADH homologs encoded by the aldH genes probably can be induced by L-arabinose and complete the arabinose degradation in these strains [118].

Glycerol is assimilated by B. cepacia strains ATCC 17759 and IPT 438, Burkholderia sp. AB4 and P. sacchari LMG 19450T for PHB synthesis [119,120]. The glycerol catabolic pathway is conserved in the Burkholderia s.l. strains (Figure 4). Glycerol is transported into the cell by a facilitator transporter (GlpF) and phosphorylated by glycerol kinase (GlpK) to yield glycerol-3-phosphate (G3P). G3P is transformed by glycerol-3-phosphate dehydrogenase (GlpD) to dihydroxyacetone phosphate, which is funneled into the lower EMP pathway.
Figure 4. Genomic comparison of the metabolism for the conversion of sugars and glycerol into PHA by *Burkholderia* sensu lato (s.l.) strains. Enzymes functions and their respective coding genes are listed above and below the graph, respectively. Numbers shown in parenthesis following the enzyme activity correspond to reactions represented in Figure 2. Color intensity depicts the gene copy number as showed in legend. Homology prediction of 27 strains was performed using the curated metabolic networks of the Kyoto Enzyme and Genomes Database (KEGG) and manual Blast search through Bidirectional Best Hit approach (BBH). For the 10 strains belonging to novel genera (e.g., *Caballeronia*, *Trinickia*, *Mycetohabitans*, *Robbsia*) a manual reconstruction through BBH was performed. An amino acid sequence identity of >30% and ≥70% coverage was used as threshold in function of the gene context for homology prediction. Genomes were retrieved from Refseq database. Strains are arranged according to a phylogenomic tree constructed from a concatenate of 38 core genes present in the 37 genomes using Phylophlan and MAD root softwares. Scr-EII, sucrose transporter of the phosphoenolpyruvate system (PTS) family; Scr-6PH, sucrose-6-phosphate hydrolase; MalL, α-glucosidase; FFast, β-fructofuranidase; MDH, mannitol dehydrogenase; GLK, glucokinase; glu-EII, glucose PTS family transporter; FK, fructokinase; G6P, glucose-6-phosphate (G6P) isomerase; 6FK, 6-phosphofructokinase; FBPase, fructose-1,6-phosphatase; FBA fructose-1,6-biphosphate aldolase; TIM, triosephosphate isomerase; fru-EII, Fructose PTS family transporter; 1PFK, 1-phosphofructokinase; G6PDH, G6P dehydrogenase; 6PGL, 6-phosphogluconolactonase; 6PGntD, phosphogluconate dehydrogenase; R5PE, ribulose-5-phosphate epimerase; R5PI, ribulose-5-phosphate isomerase; TKT, transketolase; TAL, transaldolase; GntK, gluconate kinase; PGDT, phosphogluconate dehydratase; KDPGntA, 2-keto-3-deoxyphosphogluconate aldolase; XI, xylose isomerase; XK, xylulokinase; GK, glycerol kinase; G3PDH, glycerol-3-phosphate dehydrogenase; LADH, L-arabinose-1-dehydrogenase; AL, arabinolactonase; AD, arabonate dehydratase; LKDADT, L-KDA dehydratase; KGSADH, α-ketoglutarate semialdehyde dehydrogenase.

3.2. Metabolism of Fatty Acids and PHA Synthesis in Burkholderia Sensu Lato

Different metabolic pathways may supply intermediates for PHA copolymers synthesis. These pathways include fatty acid β-oxidation and de novo synthesis (Figure 3), whose metabolic intermediates are converted into R-3HA-CoA for its subsequent polymerization by PHA synthase into poly(3-hydroxybutyryl-CoA-3-hydroxyacyl) (P(3HB-co-3HA)) or PHB [121].
Figure 5. Genomic comparison of the metabolism for the conversion of fatty acids into PHA by *Burkholderia* sensu lato strains. Enzymes functions and their respective coding genes are listed above and below the graph, respectively. Number shown in parenthesis following the enzyme activity correspond to reactions shown in Figures 2 and 3. Color intensity depicts the gene copy number as showed in legend. Homology prediction of 27 strains was performed using the curated metabolic networks of the Kyoto Enzyme and Genomes Database (KEGG) and manual Blast search through Bidirectional Best Hit approach (BBH). For the 10 strains belonging to novel genera (e.g., *Caballeronia*, *Trinickia*, *Mycehtohabitans*, *Robbsia*) a manual reconstruction through BBH was performed. An amino acid sequence identity of >30% and ≥70% coverage was used as threshold in function of the gene context for homology prediction. Genomes were retrieved from Refseq database. Strains are arranged according to a phylogenomic tree constructed from a concatenate of 38 core genes present in the 37 genomes using Phylophlan and MAD root software. ACC, acetyl-CoA carboxylase; MAT, malonyl-CoA-ACP transacylase; KAS, class I-III β-ketoacyl-ACP synthases; KR, 3-ketoacyl-ACP reductase; HAD, β-hydroxyacyl-ACP-dehydratase; ENR, enoyl-ACP reductase; ACAD, acyl-CoA dehydrogenase; HCDH/ECH multifunctional S-specific enoyl-CoA hydratase-hydroxyacyl-CoA dehydrogenase; KAT, β-ketoacyl-CoA thiolase; HB3E, 3-hydroxybutyryl-CoA epimerase; R-ECH, R-specific enoyl-CoA hydratase; PhaC, PHA synthase; PhaA, β-ketothiolase; BktB, β-ketothiolase; PhaB, acetacetyl-CoA reductase.

Fatty acid *de novo* synthesis pathway yields the intermediate R-3HA-ACP that is transformed by hydroxyacyl-ACP-CoA transacylase (PhaG) or class III ketoacyl-ACP synthase (FabH) into R-3HA-CoA, which is polymerized by PHA synthase [17,122]. All analyzed strains have a FabH enzyme (Figure 5) that probably provides PHA precursors according to a multisequence alignment analysis performed in the present review. A conserved phenylalanine residue (F97) found in FabH sequence from *Burkholderia* s.l. strains, similar to F87 in *E. coli* FabH (data not shown), suggests that FabH is specific...
VA can be obtained from LA, converted by the enzyme acyl-CoA synthetase LvaE to (C10-C16) that lead to PHA mcl-scl

Burkholderia

The closely related P. terrae and B. gladioli present review, these were not considered PhaG homologs as they are located next to rhl genes of rhamnolipid synthesis. Furthermore, PhaG enzyme has been reported to be highly similar to a β-ketoacyl reductase (RhlG) with identities of 40–45% [124]. On the other hand, the PHA mcl producer T. caryophylli AS 1.274 possesses a highly conserved enzyme with the PhaG of P. putida (75% identity) that is unrelated to rhamnolipid synthetic genes (Table 1) [125]. PhaG was not found in T. caryophylli DSM 50341 T nor in T. symbiotica JPY-345 T, therefore the presence of this enzyme is a strain-specific trait in this genus.

The metabolic intermediates of the β-oxidation of fatty acids may be funneled into PHA synthesis (Figure 3). The metabolite 2-trans-enoyl-CoA is converted into (R)-3HA-CoA by the addition of H2O to the double bond by the R-specific 2-trans-enoyl-CoA hydratase Phai or similar enzymes (MaoC, YfcX) [10,126]. In Pseudomonas aeruginosa, two phal genes encode R-specific 2-trans-enoyl-CoA hydratases with different substrate specificity [127]. The phal gene have been localized downstream of the phalP and phaC genes [128]. The closely related P. terrae DSM 17804 T, P. hospita DSM 17164 T and Paraburkholderia phynatum STM815 along with P. sacchari LMG 19450 T and Paraburkholderia spreitiae WSM5005 T have at least one phal gene copy located in a phaC gene context. While five Burkholderia sensu stricto strains and the closely related P. xenovorans LB400 T, Paraburkholderia aromaticivorans BN5 T and Paraburkholderia fungorum ATCC BAA-463 T have one copy of a fused gene coding for (R)-specific enoyl-CoA hydratase and phosphate acetyl/butyryl transferase enzymes (Phaj-Pta) (Figure 5). The precursor R-3HA-CoA may also be obtained from the β-oxidation metabolic intermediate 3-ketoacyl-CoA that is reduced by 3-ketoacyl reductase (FabG) [121]. FabG have a wide substrate specificity (C4-C12) in P. aeruginosa PAO1 [121] and the fabG gene is present in all analyzed strains in 1 to 2 copies. Finally, the intermediate (S)-3-hydroxyacyl-CoA can be metabolized by the multifunctional enzyme 3-hydroxyacyl-CoA epimerase (FabD or Fabf) into its (R)-enantionomer, R-3HA-CoA that could be polymerized to PHA scl or PHA mcl [121]. In E. coli and Bacillus subtilis the multifunctional enzymes FabD and Fabf display 3-hydroxyacyl-CoA dehydrogenase (HADH), 2-trans-enoyl-CoA hydratase (EDH) and epimerase activities [129]. These FabD and Fabf enzymes showed to be the closest relatives for B. thailandensis E264 T, P. xenovorans LB400 T and P. caeniilutica CF1 T (Figure 5). However, for the rest of the strains, the closest enzyme is FabN (also found in Cupriavidus and Bacillus strains) with the HADH and EDH conserved domains, but without epimerase activity evidence [130,131].

Valeric acid (VA) can be converted into 3-ketovaleryl-CoA, as an intermediate from β-oxidation, or into acetyl-CoA and propionyl-CoA through β-oxidation. Propionyl-CoA can be synthesized from propionic acid, pyruvic acid or levulinic acid (LA), fatty acids, threonine, methionine, valine, isoleucine and succinyl-CoA. Propionyl-CoA and acetyl-CoA may be condensed by ketothiolase (BktB) into 3-ketovaleryl-CoA, which is reduced by a ketoreductase (PhaG) into 3-hydroxyvaleryl-CoA, and that finally is polymerized by a PHA synthase into P(3HB-ketoreductase (PhaB) into 3-hydroxyvaleryl-CoA, and that finally is polymerized by a PHA synthase into P(3HB-co-3HV) [132]. The bktB gene has been identified in the majority of Burkholderia, Caballeronia and Trinickia strains with the exception of P. sacchari LMG 19450 T, P. caeniilutica CF1 T and C. sordidicola LMG 22029. Conversely, only Burkholderia stabilis ATCC BAA67 T, Burkholderia pyrrocinia DSM 10685 T and Burkholderia stagnali LMG 28156 T harbor the bktB gene of all analyzed Burkholderia sensu stricto strains (Figure 6). Additionally, VA can be obtained from LA, converted by the enzyme acyl-CoA synthetase LvaE to levulinyl-CoA (LA-CoA), and then reduced to 4-hydroxyvaleryl-CoA (4HV-CoA), which is phosphorylated to 4-phosphovaleryl-CoA (4PV-CoA). 4PV-CoA is dephosphorylated
to pentenoyl-CoA that is hydrated into 3-hydroxyvaleryl-CoA (3HV-CoA), which can be funneled into PHA biosynthesis or β-oxidation [133].

**Figure 6.** Maximum likelihood phylogenetic tree of PhaC homologues from bacteria of the order Burkholderiales. Each amino acid sequence is represented with a circle. PHA synthases from (●) Burkholderiaceae, (●) Alcaligenaceae, (●) Comamonadaceae and (●) Oxalobacteraceae, class II PHA synthases from (●) Pseudomonas, class III and IV PHA synthases from (●) Bacillus, Synechocystis, Allochromatium, Rubrobacter and Alcanivorax. Classes I-IV PHA synthases were included for comparison. Sequences were retrieved by BlastP from IMG database using class I PHA synthase from C. necator H16 as the query. Aminoacidic sequences were aligned with a progressive method (MAFFT software) and manually edited. Tree was constructed with Maximum likelihood algorithm (RaXML software), LG+G+I+F substitution model, 1000 bootstrap replicates and rooted using Minimal Ancestral Deviation (MAD) method. Groups A, B, C and D correspond to phylogenetic groups defined in Figure S1.

Burkholderia s.l. strains showed potential for the synthesis of a wide diversity of PHA_scl and PHA_mcl precursors. For example, the PHA_scl-producing strain *B. cepacia* JCM15050 that expresses the phaC gene from the PHA_mcl-producing strain *Aeromonas caviae* synthesizes P(3HB-co-3HA) [134]. Hence, PHA synthase substrate specificity along with the precursor availability are key factors that determine the monomeric composition of PHAs.

### 4. PHA Synthases in *Burkholderia* Sensu Lato Strains

PHA synthases are classified into four groups based on the primary structure, the subunit composition, and the substrate specificity [13,135]. Class I and class II PHA synthases are the most common enzymes in bacteria. Class I PHA synthases are homodimers of PhaC that are capable to polymerize mainly PHA monomers of 3–5 carbon chain-length but also polymerize medium chain length monomers (e.g., hydroxyhexanoate) [13,53,135]. The class I PHA synthase of *C. necator* H16 has been widely characterized [13,40,136]. Class II PHA synthases possess one subunit (PhaCl or PhaC2) and synthesizes PHA_mcl from precursors derived mainly from β-oxidation or the de novo biosynthesis of fatty acids [13,63,137,138]. Class II PHA synthases have been reported in diverse *Pseudomonas* species. The phaC genes encoding PHA synthases are well known mainly in *Cupriavidus* and *Pseudomonas* [64,139] and are well conserved among Betaproteobacteria and Gammaproteobacteria [74]. Class III PHA synthases are heterodimers composed of the subunits PhaC and PhaE and polymerize PHA_scl [13,39]. The Proteobacterium *Allochromatium vinosum*, the cyanobacterium...
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Synechocystis sp. and archaea such as Haloarchaea possess class III enzymes. Class IV PhaC synthases are heterodimers composed of PhaC and PhaR subunits [13,16]. Class IV PHA synthases mostly use short chain-length monomers but can also polymerize other monomers. Bacillus megaterium and Bacillus cereus class IV PHA synthases have been described [140]. In this review, we observed that class I PHA synthase is the most common type of PHA synthases in Burkholderia s.l., whereas class II PHA synthases are present in specific strains. One class III PHA synthase was found in Caballeronia grimmae LMG 27580\textsuperscript{T}, indicating that this type of synthases is not common among Burkholderia s.l.

PHA synthases contain an extended lipase box-like sequence G-G/S-X-C-X-G/A-G in the active site [135–137]. The PHA synthases of Burkholderia s.l. bacteria contain the lipase-like box sequence G-X-C-X-G-G/A. This lipase box possesses a Cys that is involved in the polymerization process by binding covalently the substrate, generating the intermediate Cys-S-3HB. The catalytic triad C-H-D, which is crucial for the activity [136], is present in Burkholderia s.l. PHA synthases (Figure S2).

The substrate specificity of the PHA synthase influences the monomer composition of the PHA. B. cepacia IPT 64 synthesizes from gluconate or sucrose a copolymer composed of P(3HB (96.5%) and poly(3-hydroxy-4-pentenoate) (P(3H4PE)) (3.5%) [141]. B. cepacia IPT64 phaC1 mutant synthesizes both homopolymers but strongly increasing the relative P(3H4PE) concentration (32%), indicating that the wild type strain possesses at least two PHA synthases with different substrate specificity [141]. B. contaminans IPT 553, which is able to accumulate P(3HB, P(3HV) and polyhydroxydecanoate (P(3HDD)) from unrelated carbon sources (i.e., glucose, sucrose glucose/casein and sucrose/casein mixture), has a class I PHA synthase [45]. The diversity of monomers synthesized by strain IPT 553 could be associated to the phaC genes. T. caryophylli AS 1.2741 possesses two class II PHA synthases encoded by two phaC gene copies separated by the PHA depolymerase encoding phaZ gene. The synthesis of PHA\textsubscript{scI} of 3HB and PHA\textsubscript{mcl} of 3HD from butyrate, and PHA\textsubscript{med} of 3HHx, 3HO and 3HD from glucose, octanoate and glucose/octanoate by T. caryophylli AS 1.2741 is reported [59,125]. E. coli KM32B expressing the phaC1 gene or phaC2 gene from strain AS 1.2741, produces PHA\textsubscript{med} of 3HHx, 3HO and 3HD from octanoate or decanoate [125]. P. sacchari LMG 19450\textsuperscript{T} synthesizes the copolymer P(3HB-co-3HHx) with a 3HHx content of 0.14–0.46% mol from glucose and hexanoate [39]. These results suggest that some PHA synthases found in Burkholderia s.l. strains, enable them to produce diverse biopolymers with different physicochemical properties, stability and availability [125,137].

To identify the phylogenetic relationships of PHA synthases in Paraburkholderia, Burkholderia, Caballeronia, Trinickia, Mycetohabitans and Robbsia genera, a survey in the available genomic data was performed. Currently, >1000 Burkholderia, Paraburkholderia and Caballeronia genomes are deposited on the comprehensive IMG/M database [142]. For the analyses of the phaC genes, we selected all complete genomes available from Burkholderia (168), Paraburkholderia (16), Mycetohabitans (1) and nine draft genomes of Caballeronia (6), Trinickia (2) and Robbsia (1) strains. The genomes of 194 strains of Burkholderia s.l. were retrieved from IMG/M database (https://img.jgi.doe.gov/index.html, accessed at 15 January 2021) and NCBI databases (https://www.ncbi.nlm.nih.gov/genome/, accessed at 15 January 2021). Then, the presence of the phaC gene was identified in the selected genomes using the BlastP algorithm provided by IMG/M database [142]. The amino acid sequence of PHA synthase from C. necator H16 (accession number P23608) was the query for carrying out the search. The proteins that displayed ≥30% amino acid identity, 70% coverage with the C. necator H16 PhaC were further studied.

All analyzed genomes possess at least one copy of the phaC gene, suggesting that all strains produce PHA. Additionally, 84% of the Burkholderia s.l. analyzed strains possess more than one phaC gene copy, indicating that phaC redundancy is a usual trait.

A preliminary phylogenetic tree delineated four distinctive groups (A, B, C, and D) of PhaC amino acid sequences from Paraburkholderia, Burkholderia and Caballeronia genomes (Figure S1). Sequences of PhaC representatives of each group obtained in Figure S1 were selected for a reconstruction of their maximum likelihood phylogeny with the PhaC found in
bacteria from the order *Burkholderiales* (Figure 6). This tree was constructed with 112 PhaC sequences from 56 *Burkholderiales* genomes (13 *Burkholderiaceae*, 16 *Alcaligenaceae*, 11 *Comamonadaceae* and 16 *Oxalobacteraceae*). Each genome harbors 1 to 6 copies of PHA synthases. Groups A and B PhaC of the *Burkholderia* s.l. strains are closely related to class I PhaC from *C. necator* H16 (Figure 6). Several of these strains are PHA<sub>scl</sub> producers [14,40,43,56]. Group C PhaC are closely related to class II PhaC from *Pseudomonas* species. PhaC found in the PHA<sub>mcl</sub> producing strain *T. caryophylli* AS 1.2741 are found in group C. Therefore, these are interesting candidates to study PHA<sub>mcl</sub> production as several of these strains that harbor group C synthases also have the metabolic pathways for the supply of PHA<sub>scl</sub> and PHA<sub>mcl</sub> precursors (Figures 3 and 5). Interestingly, groups D1 and D2 form a clade that is not related to any described class of PhaC. Strains harboring a group D PhaC produce PHA<sub>scl</sub> (PHB and P(3HB-co-3HV)) and PHA<sub>mcl</sub> (P(3HB-co-3HHx)) [39,44]. A similar finding was reported in recently isolated Janthinobacterium strains, whose PhaC2 showed a distinct clade from the known classes of PHA synthases, proposing a new PHA synthase class [143]. A multiple sequence alignment of PhaCs belonging to the groups identified within *Burkholderiales*, and model PhaC of classes I, II, III and IV was performed in order to describe key amino acidic residues described in previous structural analyses of Wittenborn et al., [136] and Kim et al., [144] (Figure S2). The substrate binding residues I252, L253, T393 and T397 and substrate tunnel residues Y445, I482 and V483 of class I PhaC are highly conserved within groups A and B, while class II and group C PhaC are less conserved in these sites. Conversely, groups D1 and D2 showed to be poorly conserved in relation to classes I-IV at these sites. These findings show that PHA synthases are more diverse than previously thought and suggests a possible new class of PHA synthases in the analyzed *Burkholderia* s.l. strains and within the *Burkholderiales* order (Figures 6 and S2).

5. Gene Synteny of the phaC Gene Cluster in *Burkholderia* Sensu Lato

Gene cluster organization of representative phaC genes of the selected *Burkholderia* s.l. identified in Figure S1 are shown in Figure 7 arranged according to their phylogenetic placement (Figures 6 and S1). Species arranged in group A have the phaCABR gene cluster organization with a close relation of PHA synthases from class I (*C. necator* H16). Remarkably, 191 of the 194 reviewed genomes of the preliminary phylogenetic analysis (Figure S1), harbor the phaCABR gene cluster, which is the most frequent pha gene arrangement. The PHA synthases encoded in the phaCABR clusters possess ~60% amino acid identity with *C. necator* H16 class I PHA synthase, which carry the same gene cluster. PHA-producing strains belonging to *P. sacchari* LMG 19450<sup>T</sup>, *P. xenovorans* LB400<sup>T</sup>, *B. thailandensis* E264<sup>T</sup> and *B. cenocepacia* J2315 possess this gene organization. Interestingly, Hiroe et al. [145] constructed plasmids with different pha gene configurations from strain H16 (phaABC, phaACB, phaBAC phaBCA phaCAB and phaCBA), using *E. coli* DH5α as chassis for biomass and PHB production analysis. Notably, strain DH5α carrying the non-natural phaCBA genes arrangement showed the highest PHB production. Nevertheless, the strain that harbor phaCAB genes configuration, which is the most typical gene organization in *Burkholderia* s.l. species, displayed the second highest PHB production. This suggests that natural strains have been selected a gene configuration that favors higher PHB and biomass yield, and synthesis of relatively low-molecular-weight polymers [145]. No functional evidence for PHAs synthesis has been described so far in the novel *Caballeronia*, *Mycetohabitans* and *Robbsia* genera. However, all strains reviewed have one phaC gene belonging to the group A, strongly suggesting that *Caballeronia*, *Mycetohabitans* and *Robbsia* strains synthesize PHA (Figure 7).
Figure 7. Gene contexts related to \( \text{phaC} \) gene of representative bacteria of the \textit{Burkholderia} sensu lato group. \( \text{phaC} \) homologous of \textit{C. necator} H16 and \( \text{pha} \) gene clusters in which the \( \text{phaC} \) gene is present. Gene contexts are arranged according to the neighbor-joining phylogeny of their PhaC amino acidic sequence shown in Figure S1. Branches tagged with black dots indicate sequences with confirmed functionality not belonging to \textit{Burkholderia} s.l. genera that have been incorporated for additional phylogenetic comparisons, while gray dots indicate \textit{Burkholderia} s.l. species with reported polyhydroxyalkanoate synthesis. The \( \text{phaC} \) genes are in black. The function of the genes included in the clusters are indicated at the bottom. The sizes of genes are at a scale depicted in the 1 kb black bar above each group organization.

The \( \text{phaC} \) genes that belong to group B are generally in conjunction with one or two additional PHA-related genes such as the \( \text{phaP} \) gene and/or the \( \text{phaJ} \) gene (Figure 7). All the bacteria that harbor a \( \text{phaC} \) gene copy arranged in group B exhibit an additional copy of the \( \text{phaC} \) gene located in the \( \text{phaCABR} \) gene cluster of group A. In \textit{Pseudomonas} and Bacillus strains, PhaJ convert \( \beta \)-oxidation intermediate 2-trans-enoyl-CoA into (R)-3-hydroxyacyl-CoA for PHA synthesis [65,138]. The \( \text{phaP} \) gene encodes the surface protein phasin that covers PHA storage granules, playing an important role preventing coalescence of granules and regulation of particle size [146]. Due to the proximity of these genes with a \( \text{phaC} \) gene, it was proposed a possible participation of these genes in PHA synthesis, increasing the PHA diversity produced by these strains.

Remarkably, the \( \text{phaC} \) genes arranged in group C (Figure 7) encode PHA synthases that showed closer similarity with the class II \( \text{phaC} \) from \textit{P. putida} (named previously \textit{P. oleovorans}) [19]. In the genomic context of this \( \text{phaC} \) gene are located the \( \text{phaZ} \) gene that encodes the PHA depolymerase, the \( \text{phaP} \) gene and the \( \text{maoC} \) gene. The \( \text{maoC} \) gene encodes a novel enoyl-CoA hydratase, which connects the \( \beta \)-oxidation with PHA biosynthetic pathway in an \textit{E. coli} fadB gene mutant defective in fatty acid \( \beta \)-oxidation, suggesting that the MaoC enzyme could replace PhaJ [147]. These data suggest that \( \text{phaC} \) gene from group C is involved in PHA biosynthesis.

The analysis of the group D showed that two subgroups could be distinguished (Figure 7). In the group D1, the genomic context of the \( \text{phaC} \) gene possesses an unusual fused gene, which apparently is a fusion between the (R)-specific enoyl-CoA hydratase encoding \( \text{phaJ} \) gene and a phosphate acetyltransferase encoding \( \text{pta} \) gene (Figure 7). Moreover, the \( \text{ackA} \) gene that encodes an acetate kinase is present in this genomic context. The phosphate acetyltransferase (\( \text{pta} \) gene) interconverts acetyl-CoA and acetate phosphate, whereas the acetate kinase (\( \text{ackA} \) gene) catalyzes the conversion of acetate into acetyl phosphate in the acetate pathway [148]. Remarkably, the overexpression of the \( \text{pta} \) and \( \text{ackA} \) genes in \textit{E. coli} improves acetate assimilation and PHA production [149]. The close presence to the \( \text{phaC} \) gene homologue of genes encoding enzymes of acetate metabolism may have
B. contaminans which harbor 4–6 whose genomes have not been sequenced, synthesize PHA (~10 Mb) that contains six phaC gene copies in different genomic contexts. The unusual high phaC gene redundancy of P. terrae strain DSM 17804 has not been observed in any other Burkholderia s.l. strain. One DSM 17804 phaC gene copy belongs to the canonical phaCABR cluster of the group A, while another four copies are included in groups B and C (Figure 6). In addition, a high phaC gene redundancy was observed in the soil strains Paraburkholderia monticola JC2948, P. hospita DSM 17164 and C. glathei LMG 14190, which harbor 4–6 phaC gene copies in their genomes.

The bktB gene encoding a β-ketothiolase that catalyzes the condensation of acetyl-CoA and propionyl-CoA into 3-hydroxyvalerate (3HV) is present in 135/194 (70%) of Paraburkholderia monticola strains and is located near the phaCABR gene cluster. Exceptionally, the closely related P. xenovorans LB400 and P. aromaticivorans BNS5 have two copies of the bktB gene close to the phaC gene. One bktB gene is located near to the phaCABR cluster, and the other copy is close to a second phaC gene copy that belongs to the group D1 (Figure 7).

The genomic analyses indicate that diverse strains belonging to Burkholderia s.l. genera are attractive candidates for the functional study of the biosynthesis of PHAs, including possible new PhaC classes within the clade or new genes and metabolites involved in PHA synthesis. Most of the PHA genes located in the phaC gene context have been proposed before. However, additional phaA and phaB gene copies located in the phaPBA cluster were observed in the Burkholderia cepacia complex, P. hospita DSM 17164 and C. glathei LMG 14190. An additional phaPB gene cluster is present in P. xenovorans LB400, P. sprentiae WSM5005, P. terrae DSM 17804, P. phymatum STM815 and C. udeis LMG 27134. A phylogenetic reconstruction of PhaA and PhaB proteins from the phaPBA and phaPB gene clusters along with those from groups A and D (Figure 6) was carried out including representative Burkholderia s.l. strains, and other Burkholderiales and Pseudomonadales reference strains (Figure 8).
Figure 8. Phylogenetic trees of representative PhaA (A) and PhaB (B) amino acidic sequences of Burkholderia sensu lato (s.l.) strains. The phaC-related gene clusters from groups A and D of Burkholderia s.l. are shown. Search was carried out through the genomic context of PHA synthases and phasin encoding genes using Refseq and KEGG databases. Organisms of Burkholderiales (blue) and Pseudomonadales (green) orders, and sequences of BktB, FadA and FabG proteins are included as references (●). Phylogeny was reconstructed using an iterative multiple sequence alignment (Mafft software), maximum-likelihood algorithm with the LG+G+F substitution model with 1000 bootstrap replicates (RaXML software) and rooted according to minimal ancestral deviation method (MAD software).

Burkholderia s.l. possess 1–2 gene copies encoding for the PhaA ketothiolases located in two different gene clusters, the phaCABR of group A (Figure 7), and the phaPBA cluster harboring a phasin-coding gene (phaP). These PhaAs amino acid sequences grouped according to the taxonomic relation of these organisms rather than to the cluster type, suggesting a vertical inheritance. Interestingly, P. xenovorans LB400 and P. aromaticivorans BNS possess a BktB that is clustered in a different branch than the BktB1 clade, closer to Cupriavidus and Pseudomonadales bacteria (Figure 8A). The PhaB ketoacyl-CoA reductase genes are located in five gene clusters: the phaCAB of group A; the phaBC-pta-ack-ahd of group D1; the phaC-pta-ack-fabI-phaB of group D2 (Figure 7); and the phaPB associated phaPBA gene clusters. In contrast to PhaA, these PhaBs are grouped according to the gene cluster type rather than the strain taxonomic relationship, suggesting a horizontal gene transfer event. The absence of the phaA gene in several pha gene clusters indicate the relevance of the PhaB presence and diversity, and the loss of the ketothiolase encoding gene or its late entry into the gene clusters [74]. This could be attributed to the physiological role of the PhaB enzyme through NADPH-mediated regulation of PHA metabolism [113]. The PhaP encoded in the phaPBA and phaPB gene clusters are interesting proteins for studying regulatory and evolutionary issues of the PHA metabolism. The regulation of the PhaP expression has been studied in C. necator H16 [157], revealing that transcriptional control is achieved by an autoregulated repressor, which is encoded by the phaR gene, having homologues located in the canonical phaCABR gene cluster included in group A (Figure 7) and located in nearly all Burkholderia s.l. strains (Figure S1). Under cultivation conditions not permissive for PHA biosynthesis in C. necator H16, PhaR binds to two sites upstream of the phaP gene and represses its transcription [157]. An analysis of the putative regulatory regions [158] upstream of the phaP gene in 8 selected Burkholderia s.l. strains and C. necator
H16 allows the identification of a 57 bp-conserved motif (Figure S3). This motif mainly overlaps both PhaR-binding sites in C. necator H16, which match the transcriptional start site plus the −10 region and a region immediately upstream of the −35 region of the σ^70 promoter of the phaP gene [157]. The identification of this conserved motif allows to predict that the regulation of PHA biosynthesis in Burkholderia s.l. strains would mirror that described in C. necator H16, involving the phaP promoter and the PhaR transcriptional repressor. Concerning the evolutionary issue, the well conserved canonical phaCAB gene cluster allows to infer that these genes were inherited from a recent common ancestor of Burkholderia s.l. strains. This is supported by the topology of a phylogenetic tree based in the concatenated amino acidic sequences of PhaC, PhaB and PhaA including the 37 Burkholderia s.l. genomes widely used in this review (Figure S4), which shows a strong consistency with the phylogeny of 38 concatenated core genes shown in Figure 1A [159–163]. The only relevant differences among the topologies based in the phaCAB genes or core genes are the inclusion of M. rhizoxinica HKI 454^T in the clade of Paraburkholderia species and the exclusion of the phytopathogens B. plantarii ATCC 43733^T and B. glumae LMG 2196^T from the Burkholderia clade (Figure S4). In any case, the strong conservation of the phaCAB gene cluster among Burkholderia s.l. species reveals the relevance of PHA biosynthesis for the fitness of this metabolically versatile proteobacteria in different ecological niches regardless of the specific lifestyle of each member.

6. Conclusions

Burkholderia sensu lato strains synthesize PHA homopolymer and copolymers from different sugars and fatty acids. In this review, the reconstruction of the metabolic pathways of 37 type and representative strains from the Burkholderia sensu stricto, Paraburkholderia, Caballeronia, Mycetohabitans, Trinickia and Robbsia genera involved in the conversion of sugars and fatty acids into PHAs was performed based on their genome analyses and previous reports. These strains possess the genes to metabolize sugars and fatty acids and related substrates into PHA_{homop} homopolymer and PHA_{scl} or PHA_{scl-mcl} copolymers. In Burkholderia s.l. strains, the ED and PP pathways but not the EMP pathway are essential routes for the conversion of sugars and related compounds into PHAs. The β-oxidation of fatty acids and fatty acid de novo synthesis are linked with the synthesis of PHAs in Burkholderia s.l. strains. Paraburkholderia and Caballeronia strains exhibited overall higher gene redundancy in carbohydrate and fatty acid metabolism than the rest of Burkholderia s.l. strains. The analysis of 194 Burkholderia s.l. genomes revealed that all these strains have the phaC gene, generally, in two or more copies. The PHA synthases of Burkholderia s.l. strains belong to the PHA synthases of class I, II, III and an outlier, and were classified into four phylogenetic groups. Four main pha gene organizations were observed in Burkholderia s.l. strains. Finally, this review describes genetic determinants related to environmental stress resistance that could be linked to PHA synthesis in Burkholderia s.l. genera. The genome analyses indicate that diverse Burkholderia s.l. strains are attractive candidates to study the synthesis of diverse PHAs.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9061290/s1. Table S1. Assimilation of substrates commonly used for PHA production in 37 Burkholderia sensu lato type and representative strains. Figure S1: Phylogenetic tree for PhaC of representative Burkholderia sensu lato species, Figure S2: Multiple sequence alignment of PHA synthases of four phylogenetic groups in Burkholderia sensu lato genomes, Figure S3: Conserved motif in the putative promoter sequence of the phaP gene in 8 selected Burkholderia sensu lato strains and the model PHA-producer C. necator H16 Figure S4: Evolutionary relationships among concatenated PhaA, PhaB and PhaC protein homologues encoded by the conserved canonical phaCAB gene cluster of the 37 Burkholderia sensu lato genomes.

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