Genome analysis of the metabolically versatile Pseudomonas umsongensis GO16: the genetic basis for PET monomer upcycling into polyhydroxyalkanoates

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Summary

The throwaway culture related to the single-use materials such as polyethylene terephthalate (PET) has created a major environmental concern. Recycling of PET waste into biodegradable plastic polyhydroxyalkanoate (PHA) creates an opportunity to improve resource efficiency and contribute to a circular economy. We sequenced the genome of Pseudomonas umsongensis GO16 previously shown to convert PET-derived terephthalic acid (TA) into PHA and performed an in-depth genome analysis. GO16 can degrade a range of aromatic substrates in addition to TA, due to the presence of a catabolic plasmid pENK22. The genetic complement required for the degradation of TA via protocatechuate was identified and its functionality was confirmed by transferring the tph operon into Pseudomonas putida KT2440, which is unable to utilize TA naturally. We also identified the genes involved in ethylene glycol (EG) metabolism, the second PET monomer, and validated the capacity of GO16 to use EG as a sole source of carbon and energy. Moreover, GO16 possesses genes for the synthesis of both medium and short chain length PHA and we have demonstrated the capacity of the strain to convert mixed TA and EG into PHA. The metabolic versatility of GO16 highlights the potential of this organism for biotransformations using PET waste as a feedstock.

Introduction

Polyethylene terephthalate (PET) is one of the most commonly used plastic polymers with an annual demand of approximately 33 million tonnes (Geyer et al., 2017). Even though PET bottles are used as an example of successful recycling, globally only 7% of PET produced annually is actually recycled (Forum, 2015) with a large majority of plastic waste still landfilled (Kasper, 2013; B, ; Plastics Europe, 2016). Among the options to reduce PET waste, the possibility of using microorganisms to both degrade and upcycle PET has gained attention (Narancic and O’Connor, 2017; Wei and Zimmermann, 2017; Blank et al., 2019). However, the ability to degrade PET is rare in nature (Wierckx et al., 2018; Salvador et al., 2019). PET belongs to the group of hydrolysable polymers and there are several examples of bacterial hydrolytic enzymes that were shown to break down PET into oligomers and monomers of terephthalate (TA) and ethylene glycol (EG; Wei and Zimmermann, 2017). While the emergence of ‘plastic eating’ bacteria such as
I. Ideonella sakaiensis (Yoshida et al., 2016) grabbed the attention of scientific and general audiences, there is still uncertainty about the rate of bacterial depolymerization of PET and the efficiency of PET monomer catabolism (Yang et al., 2016). Furthermore, the complete mineralization of PET to CO₂ will not encourage circularity (Wierckx et al., 2015). Recycling of PET waste into a material such as a biodegradable plastic like polyhydroxyalkanoate (PHA) creates an opportunity to improve resource efficiency by extending and diversifying the life of the material, thus contributing to a circular economy (European Commission, 2015; Wierckx et al., 2015). It is necessary to optimize the enzymatic hydrolysis of PET but also to develop efficient microbial transformation of PET-derived monomers TA and EG, arising from enzymatic degradation, into other molecules of value.

The strain Pseudomonas umsongensis GO16 was isolated from soil exposed to PET granules at a PET bottle processing plant (Kenny et al., 2008). The biotechnological potential of this strain was demonstrated by developing a process for the conversion of TA obtained from pyrolysis of PET into a biodegradable polymer, namely medium chain length polyhydroxyalkanoate (mcl-PHA) (Kenny et al., 2008, 2012). However, the metabolic basis of TA conversion into PHA was not investigated.

Since the genus Pseudomonas was first described in 1894, over 190 species have been identified to date (Peix et al., 2018). The ability of pseudomonads to thrive in soil, sediments, hot springs, extremely cold environments, air, plants, animals and others, is largely due to their tremendous metabolic versatility allowing them to cope with harsh and stressful environmental conditions (Poblete-Castro et al., 2017; Peix et al., 2018). The adaptability of the species belonging to the Pseudomonas genus to very different lifestyles has inspired the biotechnological use of these organisms as microbial cell factories for the production of chemicals, polymers, as bio-controlling agents, as well as in bioremediation (Poblete-Castro et al., 2017).

In this study, we have conducted a genome analysis of P. umsongensis GO16 and identified not only the genes responsible for TA and EG metabolism but also for catabolism of a wide range of aromatics. Moreover, we have identified the set of genes responsible for the synthesis of both short chain length (scl) and mcl-PHA, and experimentally validated the accumulation of these biopolymers from different substrates including an equimolar mixture of TA and EG.

Results and discussion

Overall genomic organization

The P. umsongensis GO16 genome characteristics are given in Table 1. The genome shows a very high similarity with the previously reported P. umsongensis DSM 16611, shown to degrade a wide range of xenobiotics, such as phenol, trinitrotoluene, xylene, polyaromatic hydrocarbons and petroleum (Furmanczyk et al., 2017). The GC content of these two strains is similar, however P. umsongensis GO16 genome is 650 485 bp longer (Table 1). The strain GO16 contains a 7.3 Mbp chromosome (GenBank: CP044409.1) and an 82 kbp plasmid named pENK22 (GenBank: CP044408.1).

The genome sequence was compared to notable organisms belonging to the same genus and for which a closed genome sequence is available (Fig. 1A). P. umsongensis GO16 possesses the largest genome of all of them, even larger than that of P. protegens Pf-5 (7 074 893 bp). The phylogenetic analysis conducted with coding sequences of 30 species of the Pseudomonas genus places GO16 as a member of the P. umsongensis species, closely related to P. mandelli and P. frederiksbbergensis (Fig. S1). Even though there is a high degree of functional conservation across species (Table S1), P. umsongensis GO16 has a distinct set of genome sections when compared P. umsongensis BS3657, which is the closest species for which a complete genome is available (Fig. 1B). The main differences, in addition to rearrangements, correspond to the insertion of segments likely resulting from horizontal gene transfer events in the chromosome of GO16. This is the case of a prophage (located in coordinates 805 892–830 810 bp); IS6 and IS2 transposons (1 212 038–1 253 316 bp), a region containing conjugative integrative elements, group II introns, IS3, IS5 and IS110 transposons (1 812 037–2 062 259 bp), IS3 transposon (2 661 452–2 775 901 bp); a region containing IS1182, IS110 transposons and an integrative element (4 806 223–4 889 799 bp); and regions with integrative elements (5 642 431–5 745 901 bp; 6 704 312–7 046 312 bp).

The plasmid pENK22 of P. umsongensis GO16 has a size of 81 914 kb and shows 99% identity with the 81 kb plasmid from Pseudomonas sp. MC1 (Ahn et al., 2017) and the 82 kb P. putida G7 NAH7 plasmid (Sota et al., 2006) (Fig. 1C). Like those plasmids, pENK22 encodes for a complete pathway for naphthalene oxidation with the genome of P. umsongensis DSM 16611. 

| Features | P. umsongensis GO16 | P. umsongensis DSM 16611 |
|----------|---------------------|--------------------------|
| Length (bp) | 7 269 974 chromosome + 81 914 pENK22 | 6 701 403 |
| GC content (%) | 59.2 | 59.7 |
| CDS | 6867 | 6152 |
| rRNA genes | 6 | 7 |
| tRNA genes | 57 | 62 |

Table 1. P. umsongensis GO16 genome features and comparison with the genome of P. umsongensis DSM 16611.
mineralization and has a genetic organization almost identical to NAH7 (Sota et al., 2006) with the exception of a gene rearrangement (Fig. 1D; Table S2).

Central metabolism

The central metabolism of P. umsongensis GO16 was reconstructed based on the annotation derived from the genome sequence (Fig. 2; Table S3). Like in most Pseudomonas species, glucose is likely metabolized by the Entner-Doudoroff pathway (ED), the Embden-Meyerhof-Parnas (EMP) pathway and the pentose phosphate pathway (PP), which produce a surplus of reducing power that allows for coping with stressful environments (Nikel et al., 2015). In this metabolism glucose is mainly transformed to gluconate in the periplasm prior to its conversion in 6-phosphogluconate. Similar to P. putida KT2440, GO16 lacks a cytoplasmic glucose dehydrogenase which could carry out that conversion inside the cell, and a phosphofructokinase that could transform fructose-6-phosphate into fructose-1,6-bisphosphate following the conventional EMP glycolytic pathway.

According to the functional annotation, the genome of GO16 has a large number of potentially redundant enzymes related to a likely diverse metabolism of lipids. There are a number of activities that could constitute a β-oxidation pathway including, for example, 23 genes annotated as 3-oxoacyl-(acyl-carrier protein) reductases, 5 genes coding for enoyl-(acyl-carrier protein) reductase III, 4 genes coding for 3-oxoacyl-(acyl-carrier-protein) synthase II, 17 genes for the β-oxidation enzyme acetyl-CoA C-acetyltransferase and 10 for 3-hydroxyacyl-CoA dehydrogenases.

Degradation of aromatics

Given that P. umsongensis GO16 was isolated using the aromatic compound TA as the sole carbon and energy source, we analysed the genome for the presence of pathways involved in the mineralization of other aromatics. This species shows remarkable metabolic versatility comparable or even larger than other members of the genus, possibly due to the above average size of the genome (Fig. 3) (Jiménez et al., 2004).
Our analysis identified a set of central pathways likely responsible for the degradation of monoaromatic molecules (Fig. 3 and Table S4). These include the ortho-pathway for degradation of catechol (cat), the 3,4-dioxygenolytic pathway for degradation of protocatechuolate (pca) and a pathway for the degradation of hydroxyquinol and hydroquinone (pdc), all of which converge in the β-ketoadipate pathway that leads to the central...
metabolism. In addition, we found putative central pathways for the metabolism of homogentisate (hmg), phenylacetate (paa), 3-hydroxyphenylpropionate (mhp) and the meta-cleavage for catechol (xyl) contained in the plasmid pENK22. Notably, the likely pcaGH and pcaIJ genes encoding, respectively, for the protocatechuate 3,4-dioxygenase and 3-oxoadipate CoA-transferase involved in protocatechuate degradation were duplicated in the genome (Table S4).

These pathways are used for funnelling a plethora of aromatic molecules towards central metabolism. We identified genes potentially involved in the degradation of benzoate, tryptophan, anthranilate and phenol leading to catechol; ferulate, vanillin, vanillate, coniferyl aldehyde, p-hydroxybenzoate (pOHB) and p-cresol in addition to terephthalate degraded via protocatechuate; phenylalanine and tyrosine metabolized via homogentisate; phenylethanol and phenylacetalddehyde mineralized through phenylacetate. Naphthalene and salicylate are metabolized through the putative meta-pathway for catechol encoded by the plasmid (Fig. 3 and Table S4). We confirmed experimentally that, in addition to TA, the GO16 strain can grow in 24 h on benzoate, tryptophan, pOHB, vanillate, protocatechuate, phenylacetate, phenylalanine and tyrosine (Fig. S2A and B). The strain exhibited low but detectable growth on the lignin derivatives p-coumarate and ferulate (Fig. S2C). Likewise, we confirmed growth when in the presence of naphthalene vapours over a period of 4 days (Fig. S2D). The strain was unable to use vanillin, 2-phenylethanol, anthranilate, p-cresol, 3-phenylpropionate and coniferyl aldehyde as sole carbon source in the conditions tested.

The pENK22 plasmid contains two putative operons for the catabolism of naphthalene, the upper pathway encoded by genes nahAa – nahD for the conversion of naphthalene to salicylate, and the lower pathway encoded by genes nahG – nahY for the conversion of salicylate to pyruvate and acetaldehyde via a meta-cleavage pathway spanning over 26.4 kb (Fig. 3; Table S4). This pathway organization was identified in several bacterial strains capable of aerobic degradation of aromatic compounds (Williams and Sayers, 1994; Sota et al., 2006).

**TA and EG metabolism**

As already mentioned, TA and EG are PET constituent monomers and can be obtained by pyrolysis or
enzymatic hydrolysis of PET (Kenny et al., 2008; Kenny et al., 2012; Wei and Zimmermann, 2017). Genome mining of GO16 revealed the presence of genes for the complete mineralization of both, and the capacity of GO16 to use EG as a sole source of carbon and energy was also validated experimentally.

![Genome organization of genes encoding enzymes that enable growth with EG and TA when used as a sole source of carbon and energy.](image)

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The operon for the catabolism of TA to protocatechuate (PCA) is 6085 bp long (Fig. 4A; Table S4). The catabolic genes are preceded by a regulator that belongs to the isocitrate lyase regulator-type (IclR-type) transcriptional regulators, in general involved in regulation of carbon metabolism, multidrug resistance, quorum sensing, etc. (Molina-Henares et al., 2006). In Pseudomonas strains IclR-type regulators are frequently involved in the regulation of the β-ketoadipate pathway (Molina-Henares et al., 2006). The regulator is followed by terephthalate 1,2-dioxygenase subunits α (tphA2) and β (tphA3), a reductase component (tphA1), a dehydrogenase (tphB) and terephthalate transporter (tphk). Similar organization of TA degradation operon was reported for Comamonas testosteroni strains YZW-D and E6, and Rhodococcus opacus, where TA is degraded to 1,2-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylic acid by the action of terephthalate dioxygenase (TphA2A3A1), followed by the activity of TphB to convert this intermediate into protocatechuate (Sasoh et al., 2006).

While a permease tphC is involved in the facilitated diffusion of TA in C. testosteroni strains, in GO16 the transport of TA is mediated by an MFS transporter of the aromatic acid:H⁺ symporter (AAHS) family (tphK in Table S4), which shows homology to the p-hydroxybenzoate transporter pcaK (Salvador et al., 2019) (Fig. 4A; Table S4).

We selected P. putida KT2440 for the recombinant expression of the tph genes identified in GO16. KT2440 has been certified as a safe microorganism and is considered a workhorse for biotransformations (Belda et al., 2016; Volke et al., 2020; Weimer et al., 2020). While P. putida KT2440 is equipped with numerous dioxygenases and can grow with a range of aromatic hydrocarbons, it cannot utilize TA. In fact, out of the four tph catabolic genes, only tphA2 and tphA1 have homologs in the chromosome of KT2440. They show identities of, respectively, 35% and 26% with the subunits BenA and BenC of the benzoate 1,2-dioxygenase. When the whole tph operon, including the transporter and the regulator, was expressed in P. putida KT2440 on plasmid pBT'T_pph it conferred this organism the ability to use TA as a sole source of carbon and energy (Fig. 4B). The growth rate of the KT2440 pBT'T_pph with TA as a sole source of carbon and energy was 0.3 h⁻¹, which is 1.7-fold lower compared to glucose as a sole source of carbon and energy (Fig. S3). The final biomass reached by KT2440 pBT'T_pph was 1 g l⁻¹, which is 1.6-fold lower than the final biomass of GO16 when TA is used as a sole source of carbon and energy (Fig. S3, Table 3). It is worth noting that the whole tph operon, including the transporter tphK and the native regulator iclR (Fig. 4A) were required to allow growth of KT2440 pBT'T_pph with TA as the carbon source (Fig. 4B). A construct lacking the transporter tphK (fragment tphRA2A3B1) failed to grow on TA as the sole carbon source. Moreover, no growth was observed in TA when the catabolic and transport tphA2A3BK genes cloned into pBT'T were expressed constitutively from the strong promoter P_tac. However, this construct showed increased biomass formation (0.5 g l⁻¹ CDW) when co-cultured in TA (4.2 g l⁻¹ corresponding to 1.96 gC l⁻¹) and pOHB (0.7 g l⁻¹, providing 0.42 gC l⁻¹) used as an inducer of the pca genes, compared to the culture with pOHB alone (0.28 g l⁻¹ CDW; Fig. 4B) (Kim et al., 2006). These results suggest the tphR can coordinate the expression of the tph genes and the downstream pca pathway required for PCA degradation in P. putida KT2440. Although a specific regulator for the expression of pcaGH in this organism has not been identified (Jimenez et al., 2002), TphR shares a 53% similarity with PcaU, another regulator of the IclR-family known to control the expression of pcaGH in Acinetobacter sp. ADP1 in response to PCA (Gerischer et al., 1998). These results show that both transport and regulation are specific for TA and cannot be replaced by genes present in the genome of P. putida KT2440.

In addition, we observed a transient PCA accumulation in the supernatant of KT2440 pBT'T_pph grown with TA as a sole source of carbon and energy (Fig. S4). These results indicated that the tph genes were functional in KT2440 and produced PCA, however further strain optimization is required to co-regulate the upper (TA to PCA) and lower (PCA cleavage) pathways.

EG is industrially produced at large volumes and it is used in a range of applications, production of PET being one of them. Aerobic metabolism of EG was described in some Pseudomonas species (Mückeschel et al., 2012; Franden et al., 2018; Orellana-Sáez et al., 2019). Functionally redundant periplasmic quinoproteins have been found to catalyse the initial conversion of EG to glycolaldehyde (Mückeschel et al., 2012; Wehrmann et al., 2017). In P. putida KT2440 this function is assigned to PedE (PP_2674) and PedH (PP_2679) (Wehrmann et al., 2017). In the next step, catalysed by cytoplasmic aldehyde dehydrogenases Pedl (PP_2680) and PP_0545 in KT2440, glycolaldehyde is converted to glycolate, followed by oxidation to glyoxylate by the activity of GlcDEF (Franden et al., 2018). All of the genes encoding the enzymes involved in the oxidation of EG to glyoxylate have been identified in the strain GO16 (Fig. 4A; Table 2).

The growth rate, carbon depletion and biomass yield were compared when GO16 was cultivated with TA, EG or glucose (Table 3, Fig. S5). EG supplied as a sole source of carbon and energy supports the biomass formation in P. umsongensis GO16. In comparison with the growth with TA, EG yields biomass 0.4 g l⁻¹ of cell dry
The catabolic pathway that allows biomass formation from EG in *P. putida* JM37 proceeds via Gcl pathway including a glyoxylate carboxylase (Mückeschel et al., 2012). While the wild type KT2440 contains the genetic capacity to form biomass from EG, it can only utilize it as an energy source (Li et al., 2019). It was shown that the repression of the *gcl* operon is the reason for this, and once this repression is removed, KT2440 can efficiently grow with EG as a sole source of carbon and energy (Li et al., 2019). In GO16 the *gcl* operon was identified (Table 2). This operon is regulated by a LysR transcriptional regulator (*ttuR*) and followed by carboxylate ligase (*gcl*), hydroxypyruvate isomerase (*hyi*), tartronic semialdehyde reductase (*glxR*), hydroxypyruvate reductase (*ttuD*) and pyruvate kinase (*ttuE*). The *gcl* operon is followed by the *gcl* operon, consisting of a regulator *glcC* and genes encoding the subunits of a glycolate oxidase, *glcDEF*.

It is worth noting that during the cultivation of GO16 with EG as a sole source of carbon and energy we did not observe the formation of EG oxidation products, glycolate, glyoxylate and oxalate, which was the case when *P. putida* KT2440 was grown in the presence of EG (Li et al., 2019).

**Table 3.** Comparison of growth characteristics of *P. umsongensis* GO16 when glucose, TA and EG were used as a sole source of carbon and energy. Three carbon sources were used in amounts to provide 1.96 gC l⁻¹. The specific growth rate was calculated for the exponential phase of growth

| Carbon source | Final biomass (CDW) g⁻¹ | Specific growth rate h⁻¹ | Specific rate of C consumption (g l⁻¹ h⁻¹) | Biomass yield (gCDW/gC) |
|---------------|-------------------------|--------------------------|-------------------------------------------|------------------------|
| Glucose       | 1.30 ± 0.06             | 0.22 ± 0.01              | 0.26 ± 0.02                               | 0.30                   |
| TA            | 1.60 ± 0.07             | 0.17 ± 0.01              | 0.18 ± 0.02                               | 0.35                   |
| EG            | 0.40 ± 0.13             | 0.09 ± 0.03              | 0.05 ± 0.02                               | 0.15                   |

PHA metabolism

PHAs are a family of biological polyesters which represented 1.2% of the global bioplastic market in 2019 (European Bioplastics, 2019). PHAs are bacterial carbon and energy storage polyesters usually accumulated as a response to stress (Rehm, 2010). They are grouped into scl- polymers, containing (*R*)-3-hydroxyalkanoic acids with four or five carbon atoms with polyhydroxybutyrate (PHB) as a typical example, and mcl- polymers of (*R*)-3-hydroxyalkanoic acids containing 6-12 carbon atoms (Sudesh et al., 2000). With over 150 known PHA monomers, (*R*)-3-hydroxyalkanoic acids, PHAs have highly diverse material properties and therefore a broad range of applications (Rehm, 2010).

*P. umsongensis* GO16 has a typical mcl-PHA synthesis genes organization, with a *phaC1ZC2D* cluster (Table 4). This cluster is well conserved among the mcl-PHA producing bacteria, and the two PhaCs encoded by it belong to class II PHA synthases typically involved in
the synthesis of mcl-PHA (Chek et al., 2019). We have identified an additional putative PHA synthase that also belongs to class II (Table 4), potentially contributing to mcl-PHA synthesis in GO16. This synthase is located outside of other PHA metabolism gene clusters, and it has no PHA related genes in its proximity.

Furthermore, the *P. umsongensis* GO16 chromosome encodes a scl-PHA synthesis pathway (Fig. 5A; Table 4). While the organization of the genes encoding a class I PHA synthase (phaCAB), acetyl-CoA acetyltransferase (β-ketothiolase; phaA), acetoacetyl-CoA reductase (phaB) is the same as in a scl-PHA model organism *Cupriavidus necator* H16, in this model organism the *phaCAB* operon is followed by the *phaR* encoding the PHA synthesis repressor (Pohlmann et al., 2006). The *phaCAB* genes of GO16 are followed by two genes encoding hypothetical proteins, and genes encoding a phasin family protein and PhaR repressor (Table 4). One of the two hypothetical proteins, positioned immediately after the acetoacetyl-CoA reductase, shows 99% identity with AraC transcriptional regulators found in *Pseudomonas* species (Gallegos et al., 1997). The second hypothetical protein from this cluster contains a DUF3141 domain, also found in PHA synthase of class III involved in scl-PHA synthesis (Batista et al., 2016; Chek et al., 2019).

While mcl-PHA accumulation is widely reported in pseudomonads, scl-PHA production is not a common characteristic of *Pseudomonas* species (Diard et al., 2002). *P. oleovorans* group including *P. oleovorans*, *P. pseudoalcaligenes*, *Pseudomonas* sp., as well as recently described *P. extremoastraught* and *Pseudomonas* sp. MPC6 are examples of *Pseudomonas* species that have the capacity to accumulate scl-PHA when grown with sodium octanoate (Diard et al., 2002; Lopez et al., 2009; Catone et al., 2014; Orellana-Saez et al., 2019). In *P. extremoastraught*, the scl-PHA, polyhydroxybutyrate (PHB) cluster *phaRBAC* is located in a genomic island of 32.3 kb containing 28 ORFs (Ayub et al., 2007). We have also identified an integrase 3 ORFs upstream of the scl-PHA synthase, as well as three transposases (4806223-4807659, 4818701-4820131, 4837609-4837980), suggesting that scl-PHA gene cluster could have been acquired by horizontal gene transfer.

We investigated the ability of GO16 to form mcl-PHA and scl-PHA using a variety of growth substrates. GO16 accumulated mcl-PHA when grown with glucose, TA, glycerol, octanoic acid, but no PHA accumulation was observed when EG was used as a sole source of carbon and energy (Fig. 5B). Typically, when PHA monomer-unrelated substrates were used, C10 was the major monomer detected with approximately 50 mol% (0.13–0.20 g l⁻¹). We observed a higher fraction of C12, up to 30 mol% (0.05–0.15 g l⁻¹) in mcl-PHA accumulated by GO16 in comparison with mcl-PHA accumulated by *P. putida* KT2440, which is also a model organism for mcl-PHA accumulation (Sohn et al., 2010; Davis et al., 2013). In addition to typical mcl-PHA monomers, C4 monomer was identified when butyrate (61 mol%; 0.11 g l⁻¹), gluconate (18 mol%; 0.10 g l⁻¹) or TA (1 mol%; 0.01 g l⁻¹) were used as the substrates. When butyrate was used, C4 was predominant monomer, with C8 (3 mol%; 0.007 g l⁻¹), C10 (19 mol%; 0.036 g l⁻¹) and C12 (17 mol%; 0.032 g l⁻¹) also present (Fig. 5B).

Besides the natural capabilities of GO16, we also tested the accumulation of PHA in KT2440 containing the pBT*TPH* plasmid when grown with TA as a sole source of carbon and energy. The biomass accumulated

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**Table 4.** Comparison of the genes encoding proteins involved in the synthesis and degradation of PHA present in *P. umsongensis* GO16 and those of related organisms. Gene codes starting with PP_, P or WP_ correspond, respectively, to *P. putida* KT2440, *Pseudomonas oleovorans* or *Pseudomonas extremoastraught*.

| Gene/Locus | Gene Product | Query cover (%) | Identity AA (%) | Reference gene |
|------------|--------------|----------------|----------------|----------------|
| *phaC1* F6476_32385 | Poly(3-hydroxyalkanoate) synthase 1 | 100 | 82 | PP_5003 |
| *phaZ1* F6476_32380 | Poly(3-hydroxyalkanoate) depolymerase | 99 | 91 | PP_5004 |
| *phaC2* F6476_32375 | Poly(3-hydroxyalkanoate) synthase 2 | 100 | 74 | PP_5005 |
| *phaD* F6476_32370 | TetR family transcriptional regulator | 99 | 77 | PP_5006 |
| *GA2* F6476_32365 | Granule associated protein | 65 | 66 | PP_5007 |
| *GA1* F6476_32360 | Granule associated protein | 100 | 61 | PP_5008 |
| *GA3* F6476_32350 | Putative granule associated protein | 98 | 57 | PP_5010 |
| *PHA synthase* F6476_07555 | Poly(3-hydroxyalkanoate) synthase | 94 | 49 | P26494 |
| *phaZ2* F6476_23055 | PHB depolymerase/s/β hydrolase | 88 | 37 | P26495 |
| *phaC3* F6476_22230 | Class I poly(3-hydroxyalkanoate) synthase | 99 | 73 | WP_042946539 |
| *phaA* F6476_22225 | Acetyl-CoA acetyltransferase | 100 | 82 | WP_010563427 |
| *phaB* F6476_22220 | Acetoacetyl-CoA reductase | 98 | 80 | WP_010563428 |
| *GA4* F6476_22205 | Granule associated protein | 97 | 66 | WP_003464225 |
| *phbF* F6476_22200 | PHA synthesis repressor | 98 | 69 | WP_010563433 |

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under nitrogen limitation was 0.36 g l\(^{-1}\) CDW, and 17% CDW (0.0612 g l\(^{-1}\)) was PHA. The major detected monomer was C10 (75 mol%), followed by C8 (17 mol%) and C12 (8 mol%). The biomass achieved by KT2440 pBT\(_tph\) was threefold lower compared to GO16 grown under the same conditions, and total PHA amount was 4.9-fold lower. These results show the potential to use the \(tph\) genes of GO16 for the upcycling of PET monomers in other bacterial species.

We assessed the potential of GO16 for the upcycling of PET by analysing the growth and PHA production of \(P.\ umsongensis\) GO16 using equimolar mixture of TA and EG as carbon sources. Under PHA non-accumulating conditions, TA and EG were depleted and a final CDW of 3.6 g l\(^{-1}\) was achieved, resulting in a total yield of 0.74 g\(_{CDW}\) g\(_{C}\)^{-1}. GO16 showed preferential utilization of TA over EG with utilization of EG beginning after complete degradation of TA (Fig. 6). After TA was completely depleted, a lag period of approximately 5 h was observed prior to commencement of EG metabolism. TA contributed 3.2 g l\(^{-1}\) CDW, with total TA utilization occurring within 10 h, resulting in a yield of 0.38 g\(_{CDW}\) g\(_{TA}\)^{-1} or 0.82 g\(_{CDW}\) g\(_{C}\)^{-1}. The consumption of EG contributed 0.4 g l\(^{-1}\) CDW, resulting in a yield of 0.2 g\(_{CDW}\) g\(_{EG}\)^{-1} or 0.42 g\(_{CDW}\) g\(_{C}\)^{-1}. A maximum specific growth rate (\(\mu\)) of 0.37 h\(^{-1}\) was observed when utilizing TA, and a 15-fold lower maximum specific growth rate (\(\mu\)) of 0.024 h\(^{-1}\) was recorded during EG utilization compared to TA. Total depletion of EG occurred within 8 h since the start of EG consumption.

Under PHA accumulating conditions, a final CDW of 1.5 g l\(^{-1}\) was achieved (Fig. 7), a twofold decrease...
compared to cultivation of *P. umsongensis* GO16 under non-limiting conditions. Nitrogen was completely exhausted by 8 h, leading to the onset of PHA accumulation. A total PHA content of 0.13 g l\(^{-1}\) (9% CDW) was achieved. A consumption rate of 0.97 g (l h\(^{-1}\)) TA was observed after nitrogen depletion (Fig. 7) with complete TA utilization occurring within 12 h. No EG consumption was observed, similar to what we have observed in shake flask experiments when EG was used as a sole source of carbon and energy under PHA accumulating conditions. The medium chain length PHA produced by *Pseudomonas* sp. GO16 from the equimolar synthetic mixture of TA and EG consisted of C\(_{10}\) (53 mol%), C\(_{8}\) (25 mol%) and C\(_{12}\) (24 mol%). These results indicate that upcycling of hydrolysed PET is possible mainly at the expense of TA, which is preferentially used over EG by *P. umsongensis* GO16.

**Conclusions**

In this work we have analysed the complete genome of *P. putida* GO16, a promising tool for the upcycling of PET hydrolysis monomers TA and EG (Tiso et al., 2020). The central metabolism of GO16 shows similarities with other species of *Pseudomonas*. For instance, glucose is most likely metabolized via the periplasmic conversion to gluconate and its complete metabolism combines the ED, EMP and PP pathways. This allows for the production of a larger surplus of reducing equivalents compared to the canonical glycolysis and it is linked to a better fitness in stressful environments including demanding bioconversions such as those required for the degradation of aromatics (Chavarria et al., 2013).

Related to this, *P. umsongensis* GO16 is capable of degrading a wide diversity of aromatics such as naphthalene, due to the presence of pENK22, and most notably TA, which is uncommon in *Pseudomonas* species. The presence of this pathway in GO16 enables the synthesis of different molecules of interest derived from the hydrolysis of PET. These include other aromatic molecules (Kim et al., 2019), but also the products of the cleavage of the aromatic ring and posterior modifications, some of which containing two or more functional groups and can be used as building blocks for synthesizing different types of polymers (Johnson et al., 2019). One example is adipic acid which could be obtained by the recombinant expression of a protocatechuate decarboxylase transforming the protocatechuate obtained from TA into catechol (Johnson et al., 2016). Catechol can then be cleaved by the chromosomally encoded catechol-1,2-dioxygenase to render the adipic acid precursor cis,cis-muconate.

Another interesting feature of *P. umsongensis* GO16 is the high abundance of genes related to the metabolism of lipids including several involved in different stages of \(\beta\)-oxidation. Also related to the metabolism of lipids, GO16 harbours genes for the synthesis of both scl- and mcl-PHA. C4 monomer was dominant when butyrate was used as a substrate. However, the other tested substrates drove biosynthesis of predominantly mcl-PHA monomers. It is worth highlighting that the proportion of longer acids (e.g. C12) is enriched in GO16 compared to other *Pseudomonas* species.

As a result of the complete sequencing and genome analysis of *P. umsongensis* GO16, we have been able to identify some key properties of this organism. In
particular, we have identified some aspects of its metabolism that make it a versatile workhorse for biotransformation using PET and its degradation products as feedstocks that could facilitate the development of biobased recycling strategies for this critical polymer with the potential for its conversion to not only one but two biodegradable polymers (scl-PHA and mcl-PHA).

Experimental procedures

Bacterial strain, medium and growth conditions

P. umsongensis GO16 (accession number NCIMB 41538, NCIMB Aberdeen, Scotland, UK) was inoculated from glycerol stock onto mineral salts medium (MSM) solidified with 1.5% agar supplemented with 4.4 g l⁻¹ (20 mM) disodium terephthalate (TA; Sigma-Aldrich, UK). MSM contained 9 g l⁻¹ Na₂HPO₄·12H₂O, 1.5 g l⁻¹ KH₂PO₄ and 1 g l⁻¹ (MSM₃) NH₄Cl. Prior to inoculation MSM was supplemented with MgSO₄ (200 mg ml⁻¹) and trace elements (per litre: 4 g ZnSO₄·7H₂O; 1 g MnCl₂·4H₂O; 0.2 g Na₂B₄O₇·10H₂O; 0.3 g NiCl₂·6H₂O; 1 g Na₂MoO₄·2H₂O; 1 g CuCl·2H₂O; 7.6 g FeSO₄·7H₂O).

To test the growth of GO16 with the volatile aromatic hydrocarbons naphthalene, a single colony was cultured overnight in glucose and then diluted 1:100 when transferred into a flask with 50 ml of M9 minimal medium supplemented with vitamins and trace elements (Harwood and Cutting, 1990). Naphthalene was supplemented via vapour phase from an open eppendorf tube containing crystals of the substrate (10 mg) and suspended above the culture. Flasks were incubated in an orbital shaker at 30°C and 200 rpm for 4 days monitoring the absorbance at 600 nm periodically. Growth was confirmed by

Fig. 7. Growth and PHA accumulation of P. umsongensis GO16 on minimal salts media (MSM₃) supplemented with an equimolar synthetic mixture of TA and EG to a final concentration of 40 mM (8.4 g l⁻¹ TA, 2.48 g l⁻¹ EG), mimicking enzymatically hydrolysed PET, in a 5 l bioreactor with a 3 l working volume at 30°C. The figure shows (A) biomass accumulation (CDW; g l⁻¹; ▲), nitrogen utilization (g l⁻¹; ♦) and PHA accumulation (g l⁻¹; ■) and (B) TA (●) and EG (×) utilization. Error bars represent the standard deviation of three biological replicates.
NH₄OH or 15% (v/v) H₂SO₄. DO and rpm were monitored at a value of pH 7 by the automatic addition of 20% (v/v) constant at 30°C by measuring the optical density of the cultures at 600 nm.

For the growth and PHA accumulation analysis, a single colony was inoculated into 3 ml of MSM₆ diffusion supplemented with various carbon sources (sodium gluconate, TA, EG, sodium butyrate, sodium octanoate, glycerol) in the amount corresponding to 1.96 gₜ / l⁻¹, and incubated for 18 h at 200 rpm and 30°C. The seed culture (1 ml) was inoculated into 250 ml Erlenmeyer flasks containing 50 ml MSM₆ media supplemented with the corresponding carbon source and incubated for 48 h at 200 rpm and 30°C. The cells were harvested by centrifugation at 5000 g for 10 min at 4°C (Benchtop 5430R centrifuge; Eppendorf, Germany) and washed with 10 ml of phosphate buffer (50 mM, pH 7). The pellets were frozen at −80°C.

For bioreactor experiments, the pre-inoculum was prepared by inoculating 250 ml Erlenmeyer flasks containing 50 ml MSM₆ with 1 ml of seed culture supplemented with 20 mM TA and 20 mM EG. Flasks were incubated in a shake incubator (New Brunswick Scientific, Innova 44; USA) for 18 h at 200 rpm and 30°C. Optical density readings (OD₅₄₀) of the seed culture was taken prior to bioreactor inoculation using a spectrophotometer (Spectrophotometer 6300, Jenway; UK) at 540 nm.

Batch fermentation experiments were carried out in a 5 l Biostat B bioreactor (Sartorius, Germany), containing 3 l of MSM₆ or MSM₆ broth supplemented with 40 mM TA and EG. The bioreactor was set up with 5% (v/v) inoculum of cells, with an OD₅₄₀ of 3.5 ± 0.49. Air was supplied at a constant rate of 3 l min⁻¹ (1 VVM) throughout the fermentation and dissolved oxygen (DO) was maintained at a minimum of 20%, via a control loop. Impeller speed was set to a minimum of 500 rpm and maximum of 1500 rpm. Temperature was maintained constant at 30°C. The pH of the culture was maintained at a value of pH 7 by the automatic addition of 20% (v/v) NH₄OH or 15% (v/v) H₂SO₄. DO and rpm were monitored online by BioPAT MFCS/win fermentation data acquisition software (Sartorius; Germany). 2 ml samples were taken in duplicate at hourly intervals for CDW, carbon, nitrogen and PHA analysis.

To analyse the dynamics of growth and carbon depletion, P. umsongensis GO16 and P. putida KT2440_tph were grown in 50 ml MSM₆ with glucose, TA or EG as a sole source of carbon and energy. The carbon and energy source was supplemented in an amount to provide 1.96 gₜ / l⁻¹. The cultures were incubated for 48 h at 200 rpm and 30°C, and samples were withdrawn at 6 h intervals. OD was measured at absorbance 540 nm (JENWAY 6300 spectrophotometer; Cole-Parmer, Staffordshire, UK) and 2 ml of supernatants were kept for carbon consumption analysis. The cells were harvested by centrifugation, freeze dried using a Labconco® (Fisher Scientific) freeze-dryer for CDW determination. The supernatant was retained, filtered and analysed by HPLC for carbon depletion.

For the purpose of the genomic DNA isolation, Lysogeny broth (LB; Sigma-Aldrich, Ireland) medium was used for the cultivation of P. umsongensis GO16. A single colony of P. umsongensis GO16 was inoculated into 4 ml of LB and cultivated for 16 h at 200 rpm and 30°C. The cells were harvested by centrifugation at 16 000 g, 5 min, 4°C (Benchtop 5430R centrifuge; Eppendorf, Hamburg, Germany). (Continued)

Genomic DNA extraction. The total DNA was extracted using Blood and Cell culture DNA midi kit (QIAGEN, Germany) according to manufacturer’s instructions for the lysis of bacteria. The quality and concentration of isolated DNA were verified by Qubit® Fluorometer (Thermo Fisher Scientific, Ireland) according to the manufacturer’s instructions.

Genome sequencing and analysis. The genome of P. umsongensis GO16 was sequenced by BaseClear BV (Leiden, NL). Using Illumina HiSeq platform and PacBio RSII platform a 300 bp paired-end library and 10 kb library respectively were prepared. This was followed by a de novo assembly of the reads and an automated gap closure using GapFiller version 1.10.

Sequencing of P. umsongensis GO16 total DNA (BaseClear BV, Leiden, The Netherlands) yielded 5 contigs. Specific primers corresponding to the ends of these contigs were designed and used to amplify fragments from the total DNA using Q5® High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA). The amplified products were cloned into pGEM™-T Easy vector (Promega, Madison, WI, USA) and sequenced (GATC Biotech, Ebersberg, Germany). The resulting sequences were assembled using DNAStar® Lasergene® Genomics Suite Software (Thermo Fisher Scientific, Waltham, MA, USA). These additional end sequences and the 5 assembled contigs were oriented, overlapped and ordered relative to the genome of the closely related P. umsongensis BS3657 strain. Each sequence was mapped to the BS3657 strain using ‘blastn’, part of the Basic Local Alignment Search Tool (BLAST) suite of software (Altschul et al., 1990). The relative genomic coordinates for each sequence’s location were then extracted and used to merge all contigs into one larger chromosome. The phylogenetic analysis was
conducted by an alignment-free genome-wide comparison of coding regions using a composite vector approach. The algorithm compared the amino acid counts of all predicted proteins and was implemented with the online tool CVTREE3 (Qi et al., 2004; Zuo and Hao, 2015). Blast atlases were generated by GVIEW JAVA package software (https://server.gview.ca; Petkau et al., 2010) by carrying out genome-wide tblastx searches between GO16 and each of the representative Pseudomonas genomes. The same approach was taken for comparisons between plasmid pENK22 and other plasmids containing naphthalene-catabolic genes. Regions on each genome reporting a BLAST hit above the threshold cut-off (80% identity, minimum HSP length of 100 bp, and expect value of 1e-10) were considered a valid match and represented in the figures. Genome alignments were generated using Progressive Mauve (Darling et al., 2004). BLAST searches were performed at the NCBI suite (https://blast.ncbi.nlm.nih.gov; Altschul et al., 1990) with the ‘blastp’ algorithm using either specific databases (e.g. P. putida KT2440) or the Swissprote database when running untargeted searches. BLAST searches against the genome of P. umsongensis GO16 were carried out locally with the Genome Workbench suite of NCBI (https://www.ncbi.nlm.nih.gov/tools/gbench/).

Generation of P. putida KT2440 pBTT_tph, pBTT_tphA2A3BA1K and pBTT_tphAA2A3BA1. Based on the available genome sequence of P. umsongensis GO16, the tph operon (1 982 772-1 988 856 in CP044409.1; 6128 bp) was synthesized (Twist Bioscience, UK) and cloned into pBT (Koopman et al., 2010) vector using NEBuilder® HiFi DNA Assembly Master Mix (NEB, UK). To allow Gibson assembly of the insert and the vector, the tph operon was amplified using the following primers RF 5'-TAT GCT TTG ATG TTG-3' and GR 5'-ACG TCG CAT GCT CCT CTA GAT TAA-3', while pBTT was amplified using V2F 5'-TCT AGA GGA GCA TGC GAC GTC GG-3' and V2R 5'-TCA GTA ATG ATG TTC ATT GTT GAC-3'. The ligation and transformation were performed as described above.

The insert pcaRtphA2A3BA1K fragment (5160 bp) that includes the native regulator, but excludes the transporter was synthesized by TwistBioscience (UK) and cloned into pBTT (Koopman et al., 2010) vector using NEBuilder® HiFi DNA Assembly Master Mix (NEB, UK). The primers used for Gibson assembly were RF 5'-CAC CCT GCA TTA ATC TAG AGG AGC ATG CGA CGT-3' and V1F 5'-CA CAG TGA GCC CCA-3'. The insert and the vector were amplified using V1F 5'-TGT GCA TGC GAC TCG CAT CTT CCT CTA GA-3', while pBTT was amplified using V2F 5'-TCT AGA GGA GCA TGC GAC GTC GG-3' and V1R 5'-TAT GCT TTG ATG TTG GG ACG GTG-3'. The ligation and transformation were performed as described above.

**Nutrient analysis**

For the analysis of TA consumption, the supernatant collected during cultivation had to be diluted so that the concentration of TA in the final preparation did not exceed 0.63 g l⁻¹. A 1100 series HPLC (Agilent, USA) equipped with a C18 ODS Hypersil column (150 x 3 mm, particle size 5 μm; Thermo Scientific, USA) was used, and samples were isocratically eluted using 0.4% formic acid at a flow rate of 1 ml min⁻¹ and read on a UV-vis detector at 230 nm. The TA retention time under the above conditions was 3.2 min. The PCA retention time under the above conditions was 3.2 min.

EG depletion was monitored using an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm, particle size 9 μm; Bio-rad). The column was maintained at 40°C and samples were isocratically eluted using 0.014 N H₂SO₄ at a flow rate of 0.55 ml min⁻¹ and read on a
PHA extraction and content determination. The polymer content was assayed by subjecting the lyophilized cells to acidic methanolysis as previously described (Lageveen et al., 1988). The PHA monomers’ methylesters were assayed by GC using a Hewlett-Packard 6890N chromatograph equipped with a HP-Innowax capillary column (30 m × 0.25 mm, 0.50µm film thickness; Agilent Technologies) and a flame ionization detector (FID), using the temperature programme previously described (Lageveen et al., 1988). Total PHA content was determined as a percentage of CDW.

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Conflict of interests

Author Shane Kenny is employed by the company Bioplastech. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

TN, SK, KO and JJ designed the study and supervised the research. TN, NB, SK, JU, UA and MS conducted experimental work. TN, MS, GH, HW and JJ performed the bioinformatic analyses. All authors contributed to writing the manuscript.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Blast atlas of *P. umsongensis* GO16 chromosomal genes. The table shows the results of the tblastx analysis comparing the annotated features present in the chromosome of *P. umsongensis* GO16 against the genomes of representative *Pseudomonas* species (see the experimental procedures section for details on the computational analysis).

**Table S2.** Blast atlas of the plasmid pENK22. The table shows the results of the tblastx analysis comparing the annotated features present in the plasmid pENK22 against representative similar plasmids (see the experimental procedures section for details on the computational analysis).

**Table S3.** Blast comparison of *P. umsongensis* GO16 putative genes belonging to the central metabolism. The analysis was conducted using the tblastx feature of the blast suite at NCBI (see the experimental procedures section for details on the computational analysis).

**Table S4.** Blast comparison of *P. umsongensis* GO16 putative genes taking part in the metabolism of aromatics. The analysis was conducted using the blastp feature of the blast suite and the genome workbench tool at NCBI using known enzymes for comparison (see the experimental procedures section for details).

**Fig. S1.** Phylogenetic analysis of different members of the *Pseudomonas* genus. Genome-wide comparison between *P. umsongensis* GO16 and notable *Pseudomonas* species was conducted using a composite vector approach (see the experimental procedures section in the main text for details).

**Fig. S2.** Growth profile of *P. umsongensis* GO16 in different soluble aromatics as the sole carbon source. In panels A to C cells were cultured in the microplate reader using the indicated aromatic substrates at a final concentration of 5 mM. Panel D shows bacterial growth in flasks in the presence of naphthalene vapours as the sole carbon source (see methods for details). Results correspond to the mean and standard deviation of three biological replicates.

**Fig. S3.** Growth profile and substrate consumption of *P. putida* KT2440 expressing the tph genes from *P. putida*. **Microorganisms.** Wittmann, C., and Liao, J.C. (eds.). Weinheim: Wiley-VCH, pp. 299–326.

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**umsongensis** GO16. *P. putida* KT2440 was transformed with the plasmid pBT’T tph (KT_tph) or the empty control pBT’T (WT in the plot) for comparison. Only KT_tph was able to use TA as the sole carbon source for growth (shown in red; solid line for growth and dashed line for substrate consumption). Expression of the tph genes (green lines) did not affect growth in glucose compared to the control (blue lines). Results correspond to the mean and standard deviation of three biological replicates.

**Fig. S4.** Terephthalic acid (TA) depletion and protocatechuate (PCA) accumulation in the supernatant of *P. putida* KT_tph grown with TA as a sole source of carbon and energy. (A) Kinetics of PCA accumulation and TA consumption when the strain was cultivated in MSM medium without nitrogen limitation (full nitrogen), or under polyhydroxyalkanoate (PHA) accumulating conditions (limited nitrogen) determined by HPLC-UV. (B) After 12 hours of incubation in TA and N limited conditions the culture exhibits a characteristic purple colour corresponding to PCA accumulation in the supernatant (left flask). The culture with full nitrogen that does not accumulate PCA is shown for comparison (right flask). (C) Chromatograms of TA and PCA determination.

The upper panel represents a supernatant after 12 hours of culturing in N limited conditions. Mid and lower panels represent, respectively, a standard of 0.075 g L-1 of PCA and a standard with a mixture of 0.08 g L-1 of PCA and 0.11 g L-1 of TA.

**Fig. S5.** CDW (blue lines) and substrate consumption (red lines) of *umsongensis* GO16 growing on TA (upper), EG (mid) and glucose (lower panel). All cultures contained 1.96 gC L-1. Plots show the mean and standard deviation of three biological replicates.

**Fig. S6.** Analysis of the stability of the plasmid pBT’T tph in *P. putida* KT2440. The plasmid was purified from KT2440 grown on TA as the sole carbon source using a standard miniprep protocol. The plasmid preparation was digested with SphI (lane sphI) rendering the expected fragments of 6.3 and 3.8 kb. The plasmid was also used as a template for PCR reactions with oligonucleotides V1F and V1rR annealing on the backbone and producing a 3.9 kb DNA fragment (lane pBT’T), and with oligonucleotides RT and GR that render a 6.1 kb DNA product (lane RT). Size in bp of the molecular weight markers is shown for comparison.