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Biochemistry, genetics and biotechnology of glycerol utilization in *Pseudomonas* species

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Summary

The use of renewable waste feedstocks is an environment-friendly choice contributing to the reduction of waste treatment costs and increasing the economic value of industrial by-products. Glycerol (1,2,3-propanetriol), a simple polyol compound widely distributed in biological systems, constitutes a prime example of a relatively cheap and readily available substrate to be used in bioprocesses. Extensively exploited as an ingredient in the food and pharmaceutical industries, glycerol is also the main by-product of biodiesel production, which has resulted in a progressive drop in substrate price over the years. Consequently, glycerol has become an attractive substrate in biotechnology, and several chemical commodities currently produced from petroleum have been shown to be obtained from this polyol using whole-cell biocatalysts with both wild-type and engineered bacterial strains. *Pseudomonas* species, endowed with a versatile and rich metabolism, have been adopted for the conversion of glycerol into value-added products (ranging from simple molecules to structurally complex biopolymers, e.g. polyhydroxyalkanoates), and a number of metabolic engineering strategies have been deployed to increase the number of applications of glycerol as a cost-effective substrate. The unique genetic and metabolic features of glycerol-grown *Pseudomonas* are presented in this review, along with relevant examples of bioprocesses based on this substrate – and the synthetic biology and metabolic engineering strategies implemented in bacteria of this genus aimed at glycerol valorization.

Introduction

Contemporary synthetic biology and metabolic engineering offer the possibility of expanding the substrate range of microbial cell factories beyond the sugars typically used as carbon sources (Calero and Nikel, 2019; Prather, 2019). Examples of this sort of metabolic manipulation for broadening substrate ‘palatability’ of bacteria include several chemical species, ranging from simple C1 compounds such as CO₂ or HCOOH (Antonovsky et al., 2016; Yishai et al., 2018) to structurally complex substrates such as lignocellulosic materials derived from biomass (Beckham et al., 2016; Barton et al., 2018; Kim and Woo, 2018). Alcohols conform a special category of alternative substrates for biotechnology, and they are currently being discussed as promising renewables for sustainable bio-production (Stowell et al., 1987; Smith, 2004; Dahod et al., 2010; Hoffmann et al., 2018). Glycerol (1,2,3-propanetriol, C₃H₈O₃), for instance, is a widely available, versatile and structurally simple compound that can be used as a carbon source or as a precursor in a variety of chemical and biological conversions. This polyol has been traditionally used in multiple industrially relevant areas, e.g. as an ingredient in foods and beverages (by exploiting its sweetening properties; in fact, the name *glycerol* is derived from the Greek γλυκέρος, ‘sweet’), as well as pharmaceuticals and cosmetic products, both as solvent and humectant (Pagliaro and Rossi et al., 2008b).

Biodiesel is a fuel comprised of monoalkyl (methyl, ethyl or propyl) esters of long-chain fatty acids derived...
from vegetable oils or animal fats (Hollinshead et al., 2014). Its value as a fuel has been recognized as early as the 19th century: the transesterification of a vegetable oil catalysed by a base was conducted four decades before the first diesel engine became functional (Henriques, 1898). Biodiesel has promising lubricating properties and cetane ratings compared to low sulfur diesel fuels, with a calorific value of about 37 MJ kg⁻¹. The current transesterification process used for biodiesel production involves the treatment of yellow grease (recycled vegetable oil), virgin vegetable oil or tallow with a mixture of NaOH or KOH and CH₂OH (van Gerpen and Knothe, 2010). The main by-product of this production process is glycerol: ca. 10 kg of crude glycerol is generated for every 100 kg of biodiesel produced. The fast development of the biofuel industry in several countries over the last three decades (with a global production volume of 3.8 million tons in 2005) has generated a considerable amount of crude glycerol (Suppes, 2010). Approximately 85% of all the biodiesel production over the last decade came from the European Union (Ntziachristos et al., 2014). In addition, the bioethanol process (using Saccharomyces cerevisiae as biocatalyst) generates glycerol up to 10% of the total sugar (usually sucrose) consumed in the fermentation (Hasunuma and Kondo, 2012; Mohd Azhar et al., 2017). As a consequence of this global situation, the last 10 years have witnessed the rise of glycerol as a very attractive substrate for bacterial fermentations (Mota et al., 2017). The excess of crude glycerol produced in the biofuel industry led to a decrease in glycerol price, and some years ago, it was even considered a waste (with an associated disposal cost) by many biodiesel-production plants. Converting crude glycerol into value-added products thus became a relevant need to improve the viability of the biofuel economy (Pagliaro and Rossi et al., 2008a), and both chemical and biological approaches have been explored to convert glycerol into more valuable products. Considering that crude glycerol is a non-edible renewable, its use has also advantages in terms of sustainability as it does not compete with other substrates that could be otherwise used in the food industry (Stichnothe, 2019). Compared to chemical routes for transformation of the polyol, biological transformation offers several advantages, ranging from less energy use (thus making the process more environment-friendly) to higher specificity, and increased tolerance to impurities such as salts and CH₂OH, both of which occur at high levels in crude glycerol (Katryniok et al., 2009). Over the last few years, however, global markets have changed and oil prices have stabilized—which has directly impacted biodiesel production (Pagliaro, 2017). Nevertheless, glycerol continues to attract attention as a substrate for biotechnology as it can be used by a myriad of microorganisms for the synthesis of a wide range of bioproducts (da Silva et al., 2009; Dobson et al., 2012; Pettinari et al., 2012; Mattam et al., 2013; Milrea et al., 2017). Moreover, current trends indicate that biodiesel will become the clean liquid fuel of choice in many countries, especially in those that have legal requirements to use alternatives to petrochemical fuels (Guo and Song, 2019). The United States Environmental Protection Agency, for instance, established a fuel standard volume requirement for biodiesel of 8 million litres for 2019 (Weaver, 2018) – requirements that will inevitably result in an increasing availability of raw glycerol.

Interestingly, the biotechnological value of glycerol as a substrate has been recognized since the early times of industrial microbiology (Johnsson, 1947; Gunsalus et al., 1955). In fact, some of the oldest examples of technical-scale bioreactor fermentations include the transformation of glycerol into biomass and reduced biochemical products. Nakas et al. (1983), for instance, described the fermentation of glycerol by Clostridium pasteurianum in an attempt to obtain a marketable product [a mixture of n-butanol, 1,3-propanediol (1,3-PDO) and ethanol] from glycerol photosynthetically formed by halophilic Dunaliella algae. Due to the more reduced nature of the carbon atoms in glycerol as compared to sugars (e.g. glucose and xylose, customary substrates in bioprocesses), the polyol is mostly processed via oxidative metabolism in aerobic processes. There are, however, several bacteria that can ferment this substrate anaerobically, e.g. some clostridia and a few enterobacteria (Hatti-Kaul and Mattiasson, 2016) – a circumstance that has been also exploited for the design of industrial bioprocesses. Until the last decade, for instance, it was widely accepted that Escherichia coli was unable to use glycerol as a substrate in the absence of external electron acceptors (Booth, 2005). Since then, several studies describing the fermentation of glycerol by different wild-type or mutant E. coli strains have paved the way for the efficient use of this low-cost, readily available substrate to synthesize a variety of biotechnologically relevant products under different oxygen availability conditions (Yazdani and González, 2007; Murarka et al., 2008; Nikel et al., 2006, 2008a, 2010a) – thus increasing the sustainability of fermentation processes using this polyol as the substrate. The higher degree of reduction of glycerol (γ = 4.7) over glucose (γ = 4) facilitates the synthesis of reduced bioproducts as demonstrated in E. coli strains (Nikel et al., 2008a,b, 2010b). Since less carbon has to be oxidized into CO₂ to generate reducing power, the use of glycerol potentially offers higher yields on substrate than when using sugars. Yet, what are the biotechnological uses of glycerol beyond the so-called model bacterial species?
The last decade has witnessed an exponential increase in the number of studies exploiting *Pseudomonas* species as biocatalysts. In particular, *P. putida* KT2440, a non-pathogenic soil bacterium that has been adapted to laboratory conditions (Nelson et al., 2002; Belda et al., 2016), has emerged as the chassis of choice for engineering biochemical pathways while exploiting its intrinsically high tolerance to different types of physicochemical stresses (Poblete-Castro et al., 2012a, 2017; Nikel et al., 2014a,b; Nikel and de Lorenzo, 2018a,b; Abram and Udaondo, 2019). Several studies have described the use of glycerol by *Pseudomonas* species, and biochemical and genetic studies have disclosed a rather different metabolic operation, genetic regulation and physiological responses as compared to other bacteria. Against this background, in this article, we review our current knowledge on the use of glycerol by *Pseudomonas* species either via natural or engineered pathways – with an emphasis on the physiology and metabolism of *P. putida* and the many opportunities that this substrate brings forth for biotechnological applications.

**Biochemistry and genetics of glycerol utilization by *Pseudomonas***

**General aspects of glycerol assimilation in bacteria**

In a comprehensive review of glycerol metabolism, Lin (1976) had described assimilation pathways present in several bacterial species, with a special focus on *E. coli* and related Enterobacteriaceae. Although glycerol processing in bacteria can essentially follow only two possible biochemical routes, the reduced nature of its carbon atoms renders catabolism of this substrate difficult in the absence of external electron acceptors (NO₃⁻ or fumarate). Irrespective of the pathway followed, phosphorylation and dehydrogenation steps ultimately convert glycerol into dihydroxyacetone-ᵣ⁻ (DHAP), either aerobically or anaerobically (Fig. 1). DHAP is incorporated into the central carbon metabolism as a key precursor that is further processed by the same glycolytic routes deployed when bacteria grow on sugars. Apart from the direct incorporation of glycerol-derived metabolites into biomass, this compound can be also converted into a series of reduced by-products to meet the redox and carbon balance. Bouvet et al. (1995) described bacterial species, belonging to the genera *Citrobacter*, *Enterobacter* and *Klebsiella*, capable of fermenting glycerol. In these species, there is a reductive pathway for glycerol utilization, in which the substrate is firstly dehydrated by a vitamin B₁₂-dependent enzyme to form 3-hydroxypropionaldehyde that is further reduced to 1,3-PDO by an NADH-linked oxidoreductase (1,3-PDO dehydrogenase), thereby regenerating NAD⁺. The fermentation of glycerol with the concomitant formation of 1,3-PDO [and, in some cases, 1,2-propanediol (1,2-PDO)] was also described in *Lactobacillus* and *Clostridium* species (Biebl et al., 1999).

Apart from passive diffusion, glycerol uptake in bacteria is mediated by glycerol diffusion facilitators, integral membrane proteins catalysing the rapid equilibration of glycerol concentration gradients across the cytoplasmic membrane (Stroud et al., 2003). These facilitators are α-type channels that enable the diffusion of small polyols and related molecules into the cell, and these channels are known because of their exquisite substrate selectivity. They do not permit the passage of charged compounds through them, a feature essential for the maintenance of the electrochemical gradient across the membrane. Once transported, intracellular glycerol is converted to sn-glycerol-3-P by glycerol kinase (GlpK) using ATP as the

![Fig. 1. Conserved pathways for glycerol metabolism in bacteria. In most Gram-negative bacteria, such as *E. coli*, alternative catabolic pathways ultimately lead to the generation of dihydroxyacetone-P (DHAP), which is later channelled into key glycolytic intermediates via downstream metabolism. Apart from the direct, ATP-dependent phosphorylation of intracellular glycerol (glycerol₃) indicated to the left, the polyol can be oxidized into dihydroxyacetone (DHA), and then phosphorylated using phosphoenolpyruvate (PEP) as the phosphoryl donor (as shown to the right), thereby generating pyruvate (Pyr). The enzymes involved in glycerol metabolism are GlpF, glycerol facilitator (transporter); GlpK, glycerol kinase; GlpABC, (anaerobic) sn-glycerol-3-P dehydrogenase; GlpD, (aerobic) sn-glycerol-3-P dehydrogenase; GlpD, glyceraldehyde dehydrogenase; and DhaKLM, DHA kinase. O₂ denotes a reduced quinone (e.g. ubiquinone or menaquinone), which serves as a cofactor for a flavin-containing enzyme. Enzymatic steps indicated in red are independent of the presence of oxygen, whereas the two possible sn-glycerol-3-P dehydrogenation reactions are identified with different colours depending on the availability of (alternative) electron acceptors.](image-url)
phosphoryl donor. The glycerol diffusion facilitator does not recognize sn-glycerol-3-P as a substrate and this intermediate remains inside the cell, where it is further metabolized. The driving force for the uptake of glycerol is thus generated by substrate phosphorylation by GlpK (Voeggele et al., 1993). While sn-glycerol-3-P cannot leave the cytoplasm, it can be imported into the cell by the GlpT transporter, a member of the major facilitator superfamily that couples the import of sn-glycerol-3-P into the cytoplasm to the export of inorganic phosphates from the cytoplasm to the periplasm (Lemieux et al., 2004). In E. coli, sn-glycerol-3-P can be further metabolized to DHAP by either of two membrane-bound enzymes, depending on the growth conditions (Fig. 1). Under aerobic conditions, a homodimeric aerobic sn-glycerol-3-P dehydrogenase (encoded by glpD) is produced, which can accept either oxygen or NO₃⁻ as the electron acceptor (Schryvers et al., 1978; Yeh et al., 2008). Under anaerobic conditions, a different sn-glycerol-3-P dehydrogenase is preferentially expressed — this tri-heteromeric protein complex, which is encoded by the glpABC operon, channels the electrons from sn-glycerol-3-P to either NO₃⁻ or fumarate (via the quinone pool) since oxygen can no longer be used as an electron acceptor (Cole et al., 1988). Apart from the obvious role in substrate catabolism, the presumed significance of this process is the salvage of glycerol and glycerol phosphates generated by the breakdown of phospholipids and triacylglycerol (Blom et al., 2011).

Apart from these main biochemical reactions, the first to be discovered and collectively known as the glycerol and glycerophospholipid degradation pathway, E. coli K-12 possesses an NAD⁺-linked dehydrogenase, termed GldA, which is able to support glycerol fermentation (Fig. 1). Gonzalez et al. (2008) demonstrated that GldA, annotated as a dual L-1,2-PDO dehydrogenase/glycerol dehydrogenase, is involved in glycerol fermentation both as a glycerol dehydrogenase (i.e. generating dihydroxyacetone), and as a 1,2-PDO dehydrogenase, in this case regenerating NAD⁺ by producing 1,2-PDO from hydroxyacetone. GldA is also involved in methylglyoxal detoxification (Ko et al., 2005). In this branch of glycerol metabolism, dihydroxyacetone is phosphorylated into DHAP by DhaKLM, which uses phosphoenolpyruvate (instead of ATP) as the phosphoryl donor (Jin and Lin, 1984).

Metabolism of glycerol in Pseudomonas species: substrate transport, trunk and auxiliary metabolic pathways

Although the glycerol metabolism indicated in the previous section prevails in most Gram-negative species (especially in Enterobacteria), members of the Pseudomonas genus display relevant differences both in terms of the biochemical and genetic architecture of glycerol utilization. Pseudomonas species possess over 300 known and putative nutrient uptake systems, which enable them to metabolize a large number of organic compounds and inhabit many diverse ecological niches (Silby et al., 2011). The outer membrane of these bacteria acts as a semi-permeable barrier — excluding many classes of potentially toxic molecules from the cell. Nutrients use specialized water-filled channels called porins to traverse this physical barrier (Chevalier et al., 2017); the actual entry into the Pseudomonas cell is mediated by one of four classes of cytoplasmic membrane transporters as follows: glycerol/water facilitators, phosphotransferase systems, primary active transporters and secondary active transporters (Tamber and Hancock, 2003). The first GlpF transporter to be identified in a Pseudomonas species was described in P. aeruginosa PAO1 by Schweizer et al. (1997). The authors also described a second gene within the same cluster, glpK, encoding glycerol kinase — and functionally linking substrate transport with metabolism with the genomic architecture of the cluster. While the GlpT protein of E. coli is a sn-glycerol-3-Phosphonate antiporter (Lemieux et al., 2005), the GlpT transporter present in some Pseudomonas species (such as P. aeruginosa PAO1 and P. fluorescens SBW25) seems to act as a dual sn-glycerol-3-P/phosphonamidase symporter (Hirakawa et al., 2018). Such a mechanism has not been identified in P. putida KT2440.

By gathering genetic information, the pathway for glycerol metabolism was reconstructed for both P. aeruginosa and P. putida, and it was found to be similar to the set of aerobic biochemical reactions for glycerol processing in E. coli (shown in Fig. 2A for P. putida KT2440). The sequence of reactions catalysed by the ATP-dependent GlpK kinase and the ubiquinol-dependent GlpD dehydrogenase generates DHAP, serving both as the point of entry of glycerol into central carbon metabolism and the driving force for substrate transport and consumption. DHAP, in turn, is split essentially into glyceroneogenesis (via fructose-1,6-P₂) and downward catabolism (via glyceraldehyde-3-P, GA3P; see also Fig. 2A). No enzymes similar to either GlpABC or GldA of E. coli (see Fig. 1) have been identified thus far in Pseudomonas species, indicating that oxygen-dependent pathways for glycerol utilization is the preferred route in this genus (characterized by the abundance of strictly-aerobic species (Silby et al., 2011; Nikel et al., 2014a, b)).

The relatively simple biochemistry underlying glycerol utilization is reflected in a rather conserved genetic architecture of the glp genes across species, with P. putida KT2440 as an archetypal example (Fig. 2B). In strain KT2440, the genes deemed essential for glycerol metabolism are arranged in a genomic cluster that includes glpF.
(PP_1076, aquaglyceroporin), glpK (PP_1075, glycerol kinase), glpR (PP_1074, transcriptional regulator belonging to the DeoR family) and glpD (PP_1073, the main sn-glycerol-3-P dehydrogenase). Other genes with plausible roles in glycerol processing and metabolism and annotated as such in the *Pseudomonas* database are gpsA (PP_4169, encoding a soluble, NAD(P)+-dependent sn-glycerol-3-P dehydrogenase, poorly studied in microorganisms but likely involved in glycerophospholipid breakdown as a glyceroneogenic enzyme (Reshef et al., 2003)) and several glycerol/sn-glycerol-3-P acyl transferases (Winsor et al., 2016). The structural organization of the glp gene cluster is highly conserved in both *P. putida* KT2440 and *P. aeruginosa* PAO1 — although the relative orientation of the genes is inverted. Furthermore, the glp gene cluster of strain KT2440 (i.e. PP_1076 to PP_1073) exhibits a high degree of sequence identity with glpF (83%), glpK (82%), glpR (80%) and glpD (72%) of *P. aeruginosa* PAO1 (PA_3581 to PA_3584). As indicated in the next sections, there is an intimate relationship between the genetic organization of the glp genes, the transcriptional regulation exerted by the GlpR protein, and the biochemical network deployed by *Pseudomonas* when cells are growing on glycerol.

**Growth on glycerol promotes a mixed gluconeogenic and glycolytic regime in the metabolism of Pseudomonas**

With the onset of considering glycerol as a relevant substrate for biotechnological processes, several studies have examined how *Pseudomonas* species react to this compound at different levels. Nikel *et al.* (2014a) analysed the similarities and divergences in the use of glycerol by *P. putida* with respect to other bacteria by adopting a transcriptomic approach based on deep sequencing of mRNA transcripts complemented by traditional biochemical assays. The main conclusion of that study is that growth on glycerol imposes a particular metabolic response in *P. putida* characterized by the activation of both glycolytic and gluconeogenic routes (Fig. 3). The most salient features of the genome-wide response to the substrate include (i) the transcriptional upregulation of glycerol transport and catabolic genes
(i.e. the glp gene cluster), (ii) the downregulation of alternative routes for carbon processing, (iii) the activation of a general gluconeogenic response and (iv) the concomitant slow-down of activities through the tricarboxylic acid (TCA) cycle and the gluconate/2-ketogluconate loop for oxidative processing of hexoses. The glycerol-consuming status seems therefore to favour biomass build-up while preventing loss of carbon as CO₂ or during the formation of oxidized by-products [e.g. some organic acids typically produced when Pseudomonas cells are grown on sugars (Fuhrer et al., 2005)]. Apart from these general physiological features, several regulatory nodes can be identified in the biochemical network that enable efficient and tightly-controlled substrate utilization.

The Mg²⁺- and ATP-dependent phosphorylation of glycerol to sn-glycerol-3-P catalysed by GlpK is the key regulatory and rate-limiting step in glycerol utilization in E. coli (Zwaig et al., 1970). In this species, GlpK activity is modulated by multiple factors, e.g. ATP concentration, allosteric inhibition mediated by fructose-1,6-P₂, and direct inhibition by the IIAαc cytosolic component of the sugar phosphotransferase system (Applebee et al., 2011). It is plausible that some of these regulatory features are kept in Pseudomonas species – with the likely exception of the interplay with the sugar phosphotransferase system, since glucose transport in Pseudomonas proceeds through a different mechanism (del Castillo et al., 2007; Daddaoua et al., 2009; Pfüger-Grau and de

Fig. 3. The metabolism of glycerol in Pseudomonas putida KT2440 involves a combination of special processing pathways coupled to both glycolytic and gluconeogenic routes. Reactions within the upstream central carbon metabolism in strain KT2440 affected by growth on glycerol as indicated by transcriptome and metabolic flux analyses are shown in this scheme. The biochemical network sketches the main pathways involved in carbon processing along with the enzymes catalysing the corresponding conversions. In some cases, reactions have been lumped to simplify the diagram (e.g. within the non-oxidative pentose phosphate pathway), and only some isoforms of the corresponding enzymes are shown. Further metabolism of acetyl-coenzyme A (acyetyl-CoA) is indicated by a wide shaded arrow. Glyceraldehyde-3-P (GA3P) is highlighted as a key node connecting the main metabolic blocks (indicated in the diagram as ‘initial processing steps’, ‘gluconeogenesis and anabolism’ and ‘catabolism’) active in glycerol-grown cells. DHAP, dihydroxyacetone-P; PEP, phosphoenolpyruvate. The transcriptomic and fluxomic data used in this diagram have been gathered from Nikel et al. (2014a), Nikel et al. (2015b) and Beckers et al. (2016).
Lorenzo, 2014). In addition to the enzymatic regulation of the components of glycerol catabolism themselves, more general regulatory patterns are at play in central carbon metabolism.

As indicated in the previous section, DHAP is a key metabolite connecting glycerol with the core metabolism. Downstream catabolism proceeds through the processing of GA3P via the activity of GA3P dehydrogenase. The genome of *P. putida* KT2440 encodes two *bona fide* GA3P dehydrogenase isozymes, i.e. GapA (PP_1009) and GapB (PP_2149), which are easily identified given their similarity to the same enzyme counterparts in related microorganisms. Because of the reversibility of the oxidation step of GA3P into glycerate-1,3-P₂, GA3P dehydrogenase plays a pivotal role acting either on its downward mode [i.e. glycolysis, funnelling GA3P into the Embden-Meyerhof-Parnas (EMP) pathway] and in gluconeogenesis (Lessie and Phibbs, 1984). This biochemical step lies at the very core of both glycolytic and gluconeogenic metabolic pathways in many microorganisms, deciding the direction in which the carbon flow proceeds. Apart from GapA and GapB, strain KT2440 possesses two other GA3P dehydrogenase isozymes (encoded by PP_0665 and PP_3443). RNA sequencing indicated that gapB, PP_0665 and PP_3443 are transcriptionally affected by the presence of glycerol. While PP_0665 does not seem to contribute to the total GA3P dehydrogenase activity in glycerol-grown *P. putida*, in *vitro* biochemical analyses with a ΔPP_3443 derivative of strain KT2440 accredits a role for PP_3443 as the source of a GA3P dehydrogenase activity relevant for glycerol metabolism, and its cofactor dependence (NADP⁺) points to a likely gluconeogenic role (Nikel et al., 2014a).

Glycerol metabolism relies on functional and active sugar catabolic pathways in *P. aeruginosa* PAO1 (Blevins et al., 1975; Heath and Gaudy, 1978). One of the key nodes for metabolic regulation of glycerol utilization is the activity of GA3P dehydrogenase, which appears to require an active hexoses-P metabolism. Accordingly, genes encoding enzymes within the gluconeogenic branch of the EDEMP cycle and the pentose phosphate (PP) pathway in *P. putida* KT2440 were found to be transcriptionally stimulated by growth on glycerol (Fig. 3). Furthermore, since HexR (PP_1021) is a transcriptional repressor controlling genes encoding key steps of these routes, including gapA (Udaondo et al., 2018), there is a close connection between the use of sugars and glycerol as carbon sources. The metabolite 2-keto-3-deoxy-6-phosphogluconate, an intermediate of the Entner-Doudoroff (ED) pathway (Nikel et al., 2015a), acts as a specific effector of the HexR protein (del Castillo et al., 2008) – which further supports the role of an active EDEMP cycle in enabling glycerol utilization.

The characteristic growth phenotype of *Pseudomonas putida* in glycerol cultures

When *P. putida* KT2440 is grown in a minimal medium containing glycerol, the specific growth rate attained by the cultures is ca. 30% lower than that of glucose-grown cultures; conversely, the yield of biomass on substrate increases by ca. 24% (Nikel et al., 2014a). Hintermayer and Weuster-Botz (2017) simulated growth parameters of strain KT2440 *in silico* considering 57 individual carbon sources, and experimentally validated their prediction on six of them (acetate, glycerol, citrate, succinate, malate and CH₃OH). Glycerol was found to promote the highest biomass yield on substrate (0.61 C-mol C-mol⁻¹). This feature indicates that this substrate can promote high yields not only of cell components, but also metabolites and products derived from actively growing cells (i.e. primary metabolites).

A phenomenon consistently observed in glycerol cultures is a considerable lag phase (Esca et al., 2012a; Nikel et al., 2014a), which has been interpreted as the macroscopic consequence of a substantial re-arrangement of the whole metabolic network prior to reaching an optimum for growth on this substrate. Closer examination of the phenomenon revealed a stochastic transcriptional response of the *glt* genes as explained later in this article. In any case, RNA sequencing in cells harvested from these cultures indicated a general decrease in the transcription of genes encoding stress response components, further accompanied by the differential expression of elements of the respiratory chain. This raises interesting questions on the relationship between growth rate, stress and the general fitness in Gram-negative bacteria. It has been suggested that microorganisms are subjected to the general biological principle of *caloric restriction*, i.e. highly energetic carbon substrates lead to transient fast growth – but also to physiological stress and a relative loss of individual reproductive capacity (Skinner and Lin, 2010). The overall physiology of glycerol-grown *P. putida* is consistent with such a perspective: by avoiding to overrun the reactions within the TCA cycle and peripheral (oxidative) metabolic loops, and by recycling carbon equivalents to biomass building blocks, cells may grow slower in glycerol and be less energized. Yet, under these circumstances, the impact of metabolism would not be highly stressful – and the population as a whole should be eventually more successful in terms of final numbers. The fact that glycerol itself acts as an osmoprotectant (Sleator and Hill, 2002) indicates that growth on the polyol determines a less stressful cell physiology as compared to the use of sugars as a carbon source. Accordingly, the maintenance coefficient of *P. putida* KT2440 growing on glycerol has been determined to be...
Glycerol utilization analysed from a systems biology perspective

Environmental bacteria have developed remarkable regulatory systems, which allow them to thrive and cope with various environmental conditions such as extreme temperatures, exposure to metals and nutrient availability, to name but a few of them (Dominguez-Cuevas et al., 2006; Daniels et al., 2010; Krell et al., 2012; Tribelli et al., 2013; de Lorenzo et al., 2015; Belda et al., 2016; Chavarria et al., 2016). Bacteria display an exquisitely fine-tuned modulation of gene expression with the aim to maintain cellular functions, with this regulation occurring both at the transcriptional and the post-transcriptional level (Arce-Rodriguez et al., 2016). These regulatory programs control hundreds of enzymatic reactions, fuelling metabolism and sustaining bacterial growth on a variety of nutritional situations (Schuetz et al., 2012). How nutrient availability drives global gene expression in bacterial species has been an important area of study in the last decade (Chubukov et al., 2013; Kohlstedt et al., 2014; Vital et al., 2015). As indicated above, Pseudomonas species have received special attention when grown on glycerol as the sole carbon source under different fermentation modes (Wang and Nomura, 2010; Kim et al., 2013; Licciardello et al., 2017). It is important to highlight that the physiology of cells growing in batch cultures (i.e. in the presence of excess substrate) highly differs from that in continuous cultivation setups in terms of gene expression (transcriptome), protein abundance (proteome) and conversion rates in biochemical reactions (fluxome) – which ultimately define growth patterns and macroscopic phenotypes. Chemostats are an excellent tool to evaluate physiological parameters since constant growth rates can be externally adjusted by the operator through the dilution rate (D) while maintaining other relevant parameters strictly controlled (e.g. pH, oxygen levels and nutrients concentration).

Against this background, Beckers et al. (2016) recently elucidated gene expression and metabolic flux patterns in P. putida KT2440 grown on glycerol under different growth regimes and nutrient-limiting conditions. Genes belonging to the oxidative PP pathway (zwfA), ED pathway (eda) and the pyruvate node (acoABC, encoding the components of a dual dehydrogenase) showed higher expression levels by changing the imposed specific growth rate (from 0.044 to 0.12 h⁻¹) under carbon limitation conditions – echoing the results previously observed in batch cultures with glycerol (Nikel et al., 2014a). Remarkably, this was not the case for the same genes of the PP and ED pathways when their expression was examined under nitrogen limitation: when shifting from carbon to nitrogen limitation, the mRNA levels of genes of the PP and ED pathways showed no changes, and gene encoding elements of both the pyruvate node and isocitrate dehydrogenase (encoded by icd, PP_4012) were downregulated (Beckers et al., 2016); see also Fig. 3. Analysis of the metabolic flux via the PP and ED pathways corroborated the findings from the transcriptome analysis, with a strong dependence on the activity of the EDEMP cycle when cells were grown under nitrogen limitation at D = 0.12 h⁻¹.

The scenario described above is somewhat different to the one observed in P. putida LS46 grown in glycerol-containing batch cultures (Fu et al., 2015). By comparing nitrogen versus carbon limitation, the authors have found that the transcription of genes encoding pyruvate dehydrogenase and isocitrate dehydrogenase was upregulated, and proteomic analysis supported this observation at the enzyme abundance level. In addition, carbon fluxes through the glyoxylate shunt (usually inactive in the presence of glucose) were found to be extremely high under nitrogen limitation, giving rise to two industrially important by-products, succinate and malate. These
observations indicate that the pattern of metabolic regulation differs among strains, and furthermore highlight the relevance of glycerol as a substrate for the synthesis of reduced bioproducts. The next relevant question is how the overall cell physiology in glycerol-grown *P. putida* is tied to the unique transcriptional signature imposed by the GlpR regulator – an issue examined in the following section.

The transcriptional activation of the glp gene cluster follows a bimodal regime and defines the glycerol-dependent growth pattern of *P. putida*: a unique case of metabolic persistence

The emergence of methodologies designed to study bacteria at the single cell level revealed a complete repertoire of responses of individual microorganisms to specific environmental cues (Ackermann, 2015; Roberfroid et al., 2016; Osella et al., 2017). These observations challenge the customary view of prokaryotic growth and metabolism as a homogeneous, co-occurring process in space and time. The phenomenon broadly known as persistence, i.e. the occurrence of a live but non-growing fraction of cells within a bacterial population (van den Bergh et al., 2017), is a prime example in this respect. While the lack of microbial growth may appear negative at a first glance, persistence ensures the survival of cells exposed to agents hitting developing bacteria, e.g. antibiotics. Once the selective pressure ceases, persistent bacteria can resume growth and fully restore the original population (Balaban et al., 2004). Regardless of the mechanisms behind this phenomenon, the standing question is whether persistence is to be considered as an adaptive trait or a casual occurrence. What we qualify as persistence may just be a particular case of a more common situation in which a starting population stochastically splits between growing and non-growing cell types when facing a new set of environmental or physicochemical conditions (Cabral et al., 2018). Environmental bacteria are also subjected to clonal and phenotypic variability (van den Broek et al., 2005; Volke and Nikel, 2018; Schiessl et al., 2019), especially when growing on alternative substrates such as aromatic compounds (Nikel et al., 2014c) – but our studies on glycerol utilization by *P. putida* revealed that simpler carbon substrates can likewise elicit a similar stochastic response.

A noteworthy feature consistently detected in glycerol cultures is an anomalously long lag phase (typically lasting \(>10\) h) before any noticeable growth is evident (Escapa et al., 2012a; Nikel et al., 2014a) – an occurrence not observed when the cells are cultured on glucose or succinate under the same conditions. Exposure of strain KT2440 to glycerol leads to the appearance of two sub-populations that differ in their level of metabolic activity towards the carbon substrate, and the relative proportion of these bacterial sub-populations (i.e. active and inactive) changes over time (Nikel et al., 2015b). The phenomenon has been studied by defining the so-called time of metabolic response, which identifies the stretch needed for single-cell cultures to reach an optical density at 600 nm (OD\(_{600}\)) of 0.3 units, i.e. corresponding to mid-exponential growth (Nikel and de Lorenzo, 2018a). By systematically recording OD\(_{600}\) values in 1000 independent, single-cell microtitre-plate cultures of *P. putida* KT2440 grown on either glucose or glycerol, the distribution of times of metabolic response was plotted as a function of the time elapsed since inoculation (Fig. 4A) – clearly identifying the existence of more than one bacterial sub-population in glycerol cultures. Flow cytometry-assisted analysis of the overall level of metabolic activity in these cultures supported this notion: glycerol cultures were characterized by the presence of a dormant fraction of bacterial cells coexisting with a metabolically active sub-population, whereas a single, uniform and metabolically active *P. putida* population was observed when cells were grown in the presence of glucose. This phenomenon seems to represent the mirror counterpart of persistence, i.e. the stochastic rise of individual cells able to metabolize the substrate amidst a majority of glycerol-unresponsive bacteria, followed by the eventual take-over of the entire *P. putida* population.

Elucidation of the functional interactions between glycerol-derived metabolites and the transcriptional architecture of the *glp* gene cluster in strain KT2440 provided an explanation for the macroscopic phenotype of cells grown on glycerol cultures (Fig. 4B). While virtually all prokaryotic promoters are subject to a degree of noise (Elowitz et al., 2002), certain regulatory devices translate such noise into bi/multi-modal or bi-stable manifestation of the corresponding phenotypes in single cells. Cells will start growing only if the low-probability effector-independent stochastic lifting of the GlpR-mediated repression allows for the expression of *glpF* and *glpK* (the latter gene encoding the kinase responsible of sn-glycerol-3-P formation). Once this repression is stochastically defeated, the full expression of the *glp* genes can proceed – finally returning to an OFF state when the substrate is completely depleted. Different levels of metabolic activity are observed in the cells, reflecting their ability to catabolize glycerol, while the transcriptional derepression process is undergoing. This situation, in turn, explains the very long lag phase in *P. putida* cultures on glycerol. Further confirmation of this hypothesis comes from (i) deletion of *glpR* and (ii) controlled overexpression of *glpFK* – both manipulations resulting in the disappearance of the protracted lag phase on glycerol, and in the uniform distribution of growth phenotypes.
The prolonged unresponsiveness of cells exposed to glycerol could enable carbon source-dependent metabolic bet-hedging to explore new chemical and nutritional landscapes; a concept reminiscent of foraging in animal ecology, in which some members of the population (but not the entire population) take risks to broaden the search for alternative food sources. Under this scheme, the cost of randomly expressing metabolic genes in *P. putida* is outweighed by the potential benefit of locating (and being prepared to utilize) alternative carbon sources such as glycerol, which is not usually present at high concentration in environmental niches colonized by *Pseudomonas*. After discussing the intricate combination of biochemical and genetic mechanisms of regulation in glycerol-grown *Pseudomonas*, we now move onto another relevant aspect of this compound, i.e. its value as a carbon substrate in biotechnological processes.

**Biotechnology of glycerol valorization by *Pseudomonas* species**

Microbial fermentations using glycerol as the main carbon source have been exploited for the production of a...
wide variety of value-added compounds, ranging from simple molecules to structurally complex polymers (Fig. 5A). In some practical cases, glycerol has been used to promote Pseudomonas-based biotransformation processes (Fig. 5B), in which the biocatalyst executes a given biochemical reaction fuelled by the addition of a carbon source besides the substrate being transformed. While all the examples available in the literature describe aerobic processes for glycerol valorization, the possibility of engineering a micro- or anaerobic metabolism in P. putida remains a fascinating – and challenging (Nikel and de Lorenzo, 2013) – possibility that could open new avenues for biotechnological production (Fig. 5B). In the sections below and Table 1, we present some of the most relevant examples on the use of glycerol as the main substrate for the production of value-added molecules by Pseudomonas species.

**Polyhydroxyalkanoates – industrial biopolymers**

There is little doubt that one of the biggest challenges that modern society is facing is the use of non-renewable materials for the production of fine and bulk chemicals at the industrial scale (Becker and Wittmann, 2018; de Lorenzo et al., 2018; Dupont-Inglis and Borg, 2018). When glycerol emerged as a promising feedstock for bacterial fermentation in the last decade, the bioconversion of this substrate into polyhydroxyalkanoates (PHAs), a family of biopolymers with similar mechanical and physical properties to that of synthetic thermoplastics, became an immediate goal. Many bacterial species synthesize PHAs as carbon and energy storage compounds under growth conditions characterized by an abundance of carbon sources with respect to other nutrients, such as nitrogen or phosphorus (Anderson and Dawes, 1990; Gomez et al., 2012; López et al., 2015). The physicochemical properties of these polymers (e.g. thermoplastic properties, and hence, industrial applicability) largely depend on the size (i.e. chain length) of the monomer (Meng and Chen, 2018). The most common and widespread PHA is poly(3-hydroxybutyrate), but several bacteria are known to accumulate PHAs with monomers of lengths between 3 and 20 carbon atoms when fed with specific substrates (Leong et al., 2014). Polymers composed by C3-C5 monomers are called short-chain-length PHAs (scl-PHAs), whereas medium-chain-length PHAs (mcl-PHAs) contain C6-C14 monomers. Long-chain-length PHAs have monomers longer than C14. These polymers continue to attract increasing industrial interest as renewable, biodegradable, biocompatible, and extremely versatile thermoplastic and elastomeric materials (Suriyamongkol et al., 2007; Koller et al., 2017). The biochemistry and molecular biology of PHA synthesis and degradation in several bacterial species has been elucidated (Kessler and Witholt, 2001). PHAs are deposited intracellularly as complex inclusion bodies or granules (Grage et al., 2009). Polymer granules include, among other proteins, PHA synthase, depolymerizing enzymes, regulatory proteins, and structural proteins termed phasins (Mezzina and Pettinari, 2016).

**Pseudomonas species** are natural producers of mcl-PHAs (Prieto et al., 2016; Poblete-Castro et al., 2017), and these polyesters can be accumulated under nutrient imbalance conditions using a broad array of carbon sources, e.g. fatty acids, sugars, waste materials and glycerol (Poblete-Castro et al., 2014). When Pseudomonas is used as a cell factory for PHA production, the monomer composition can be tuned depending on the carbon source and the fermentation mode chosen for biopolymer synthesis (Meng et al., 2014; Chen and Jiang, 2017; Meng and Chen, 2018), since the class II PhaC polymerase enzyme of Pseudomonas accepts a broad range of substrates (Prieto et al., 2016). Once

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glycerol is converted into acetyl-coenzyme A (CoA) in several steps (Fig. 3), this intermediate is redirected to the de novo synthesis of fatty acids, resulting in various precursors for the PHA biosynthesis route (Beckers et al., 2016). One of the key enzymes of this process is PhaG (a transacylase), which converts (R)-3-hydroxyacyl-acyl carrier protein (ACP) thioesters into (R)-3-hydroxyacyl-CoA, the substrate of PHA polymerase – thus linking the de novo synthesis of fatty acids with the PHA cycle (Rehm et al., 1998; Escapa et al., 2012b). The biopolymer obtained thereby consists of a mixture of various monomers, but it was found to be particularly enriched in the 3-hydroxydecanoate (C10) fraction.

*Pseudomonas* strains can accumulate >30% of its cell dry weight as PHA when grown on glycerol (Escapa et al., 2012a), yet PHA productivities and yields are rather low as compared to those observed when fatty acids are used as substrates (Fu et al., 2014). Most studies of mcl-PHAs synthesis from glycerol have focused on the use of raw glycerol (i.e. the by-product of the biodiesel industry) as the carbon substrate. Given its high capacity to cope with toxic compounds, e.g. CH₃OH, present in raw glycerol at relatively high concentrations, *Pseudomonas* cells exhibit essentially the same growth pattern as compared to cultures containing pure glycerol. Fed-batch culture production of mcl-PHA in *P. putida* GO16 on raw glycerol resulted in a PHA titre of 6.8 g l⁻¹ after 48 h of cultivation (Kenny et al., 2012). Various *P. putida* strains have been evaluated for their capacity of producing mcl-PHA on raw glycerol, and *P. putida* KT2440 was found to be the best performer. Interestingly, citrate was observed to accumulate as a by-product in the culture broth during the fermentation period, reaching a titre of >20 g l⁻¹ in bioreactor cultivations (Poblete-Castro et al., 2014). By-product formation is certainly an undesired feature for the efficient production of biopolymers, but citrate seems to be regularly present in glycerol fermentations due to the high amount of carbon used during the process. In an attempt to increase the amount of carbon available for PHA synthesis, metabolic engineering strategies have been applied in *P. putida* with the aim of reducing by-product formation, e.g. by model-driven engineering of strain KT2440, which yielded as much as twice mcl-PHA content as compared to the parental strain (Sohn et al., 2010; Poblete-Castro et al., 2012b). In a separate study, knocking out *glpR* was shown to result in a ca. twofold increment in the mcl-PHA content produced by strain KT2440 in shaken-flask cultivations with glycerol as the carbon source (Escapa et al., 2012a). Moreover, deletion of *phaZ* encoding a PHA depolymerase, resulted in ca. 1.4-fold higher levels of mcl-PHA content than the wild-type strain when using raw glycerol as the only carbon substrate (Poblete-Castro et al., 2014). Based on elementary mode analysis, several genetic targets have recently been proposed attempting to enhance PHA accumulation in *Pseudomonas*.
putida in the presence of glycerol (Beckers et al., 2016). A novel programmable genetic circuit for cell autolysis was developed and tested in glycerol-grown in P. putida KT2440 cells when accumulating mcl-PHAs at high levels (Borrero de Acuña et al., 2017). This efficient cell lytic system was based on the heterologous expression of a peptidoglycan-disrupting enzyme lysozyme, which was further translocated to the periplasm using a signal peptide of P. stutzeri. Upon induction under nitrogen-limiting conditions, > 95% of the cell population showed membrane disruption and ca. 75% of the PHA could be recovered at the end of the fermentation period. The application of synthetic biology and systems metabolic engineering approaches in Pseudomonas strains, coupled to in-depth analysis of the phenotypic outcome of these manipulations, is expected to further boost biopolymer production from glycerol towards economic feasibility.

Rhamnolipids and other biosurfactants

Some Pseudomonas strains can thrive on water-immiscible substrates, such as alkanes and lipids, by secreting specific amphiphilic compounds called biosurfactants, which reduce the tension and interfacial surface between the immiscible substance and water (di Martino et al., 2014; Patel et al., 2019). The best-studied biosurfactants are rhamnolipids, i.e. a glycolipid in which one or two molecules of rhamnose are linked to one or two ω-hydroxydecanoate moieties (Desai and Banat, 1997; Wittgens 2014; Patel et al., 2019). The best-studied biosurfactants are rhamnolipids, which reduce the tension and interfacial surface between the immiscible substance and water. Some species of the same chemical species (Dvořák et al., 2017). Several examples from the literature indicate that P. putida has been engineered for the production of aromatic compounds that are often extremely toxic to be handled by other microbial hosts, e.g. cinnamate, p-coumarate, p-hydroxybenzoate and phenol (Calero et al., 2018). One of the pioneering works in engineering P. putida for the biosynthesis of aromatic chemicals was the construction of heterologous pathways leading to L-tyrosine and p-hydroxybenzoate using the solvent-tolerant P. putida S12 as the biocatalyst (Verhoef et al., 2007). When cells were grown on glycerol, p-hydroxybenzoate titres of 0.24 and 1.78 g l⁻¹ were attained in batch and fed-batch processes, respectively. More recently, P. taiwanensis VLB120 was engineered by knocking in and out genes of the L-tyrosine pathway and other routes of aromatic degradation (Jiménez et al., 2002), and a phenol-producing strain was obtained via the heterologous expression of an efficient tyrosinase phenol-lyase in a plasmid-free strain that bears 22 genetic modifications in total (Wynands et al., 2018). Inactivation of pykA (encoding pyruvate kinase) in the engineered P. taiwanensis strain further increased the yield of phenol on glycerol up to an unprecedented 18.5% Cmol C-mol⁻¹. The potential of Pseudomonas species as platforms for aromatic compound production from glycerol is expected to be further explored in the future, as

Production of aromatic compounds

Like many other value-added products, aromatic compounds are mainly produced from fossil and other non-renewable resources using processes that involve toxic precursors (e.g. benzene or toluene), high temperatures and complex reaction sequences (Gosset, 2009; Lee and Wendisch, 2017). Pseudomonas species have been proposed as biocatalysts for the production of aromatic compounds (Kuepper et al., 2015; Molina-Santiago et al., 2016), partially in view of the fact that members of this bacterial genus are known to be outstanding degraders of the same chemical species (Dvořák et al., 2017). Several examples from the literature indicate that P. putida has been engineered for the production of aromatic compounds that are often extremely toxic to be handled by other microbial hosts, e.g. cinnamate, p-coumarate, p-hydroxybenzoate and phenol (Calero et al., 2018). One of the pioneering works in engineering P. putida for the biosynthesis of aromatic chemicals was the construction of heterologous pathways leading to L-tyrosine and p-hydroxybenzoate using the solvent-tolerant P. putida S12 as the biocatalyst (Verhoef et al., 2007). When cells were grown on glycerol, p-hydroxybenzoate titres of 0.24 and 1.78 g l⁻¹ were attained in batch and fed-batch processes, respectively. More recently, P. taiwanensis VLB120 was engineered by knocking in and out genes of the L-tyrosine pathway and other routes of aromatic degradation (Jiménez et al., 2002), and a phenol-producing strain was obtained via the heterologous expression of an efficient tyrosinase phenol-lyase in a plasmid-free strain that bears 22 genetic modifications in total (Wynands et al., 2018). Inactivation of pykA (encoding pyruvate kinase) in the engineered P. taiwanensis strain further increased the yield of phenol on glycerol up to an unprecedented 18.5% Cmol C-mol⁻¹. The potential of Pseudomonas species as platforms for aromatic compound production from glycerol is expected to be further explored in the future, as
the wealth of catabolic pathways for these chemical structures in the *Pseudomonas* genus offers unique opportunities for engineering novel biosynthesis routes.

**Biosynthesis of other value-added chemicals**

The production of cis,cis-muconic acid ([2E,4E]-2,4-hexanedioic acid) has been the subject of intense research, as this dicarboxylic acid is a relevant platform chemical and precursor to terephthalic acid, 3- and 2-hexenedioic acid, 1,6-hexanediol, ε-caprolactam and ε-caprolactone – all of which are building blocks of commercial plastics, resins and polymers (e.g. Nylon-6,6 via adipic acid). The synthesis of *cis,cis*-muconic acid from glycerol has been explored using *P. chlororaphis*, a plant growth promoting species, as the platform strain (Wang et al., 2018). The engineering strategy consisted of blocking *cis,cis*-muconic acid conversion into end products that could be further metabolized, and augmenting the supply of metabolic precursors by overexpressing catA, the gene encoding the rate-limiting step of the pathway (catechol 1,2-dioxygenase). The resulting plasmid-free *P. chlororaphis* strain was able to synthesize *cis,cis*-muconic acid to a maximal titre of 3.4 g l\(^{-1}\) in a fed-batch process, accompanied with a product yield on glycerol of 0.19 g g\(^{-1}\). Although this performance is far below the *cis,cis*-muconic acid titre of 65 g l\(^{-1}\) achieved in metabolically engineered *P. putida* from aromatics and a co-feed of glucose (Kohlstedt et al., 2018), synthesis from glycerol remains an interesting possibility for the future if further improvements in product yield and titre are achieved.

Metabolic engineering strategies have also been applied in *P. putida* for the glycerol-dependent production of biofuels. For instance, an engineered variant of the solvent-tolerant *P. putida* S12 strain capable of producing butanol from glycerol was developed by Nielsen et al. (2009). This strain synthesized 220 mg l\(^{-1}\) of butanol in batch cultures. Although still far from the high titre reached by natural butanol-producing strains, e.g. from the genus *Clostridium*, the potential of these engineered *Pseudomonas* strains is very high due to their production performance in bioreactors and high tolerance to the toxicity exerted by biofuels to the cells (Rühl et al., 2009; Vallon et al., 2015; Cuenca et al., 2016).

Another industrially relevant chemical recently obtained from engineered *P. fluorescens* is alginate, which is widely used in both the food and pharmaceutical industry. This agent is added to food products as an emulsifier, stabilizer and texture-improver (Bonnichsen et al., 2015). In the pharmaceutical manufacturing sector, alginate is compounded into tablets to speed up their disintegration with the aim to release the medically active components in a more controllable fashion as well as to help protecting the stomach mucosa. The non-pathogenic *P. fluorescens* SBW25 ΔmucA has been reported to produce alginate from a wide variety of carbon sources such as glucose, fructose and glycerol. Disruption of this anti-β factor regulator (encoded by *PFLU_1468*) enables the transcription of algU, essential for alginate biosynthesis. This co-polymer of (1→4)-linked β-α-mannurionate and its C5-epimer α-L-gulurionate has been obtained at high titres in both batch and chemostat cultures of *P. fluorescens* SBW25 ΔmucA, reaching up to 8 g l\(^{-1}\) of polysaccharide (Maleki et al., 2017).

3-Hydroxypropionate is another important platform chemical that has received increasing attention in the last few years, given its use for the production of acrylic acid and acrylamides. Production of this compound from glycerol was obtained by overexpressing a glycerol dehydratase of *K. pneumoniae* into *P. denitrificans*, a natural producer of vitamin B12 (Lago and Demain, 1969) – which is needed for the biosynthesis of 3-hydroxypropionate from glycerol (Zhou et al., 2013). Yet, there is still a major drawback in using *P. denitrificans* as a host for engineering biosynthetic pathways for 3-hydroxypropionate since this species can consume the product (a common metabolic signature of many *Pseudomonas* species). Inactivation of the uptake system for 3-hydroxypropionate in *P. denitrificans* appears to be a straightforward strategy to advancing the production of this valuable chemical in *Pseudomonas* – an approach that has been also adopted for the production of butanol in *P. putida* (Cuenca et al., 2016).

**Conclusions and outlook**

Over the last few years, glycerol has become an appealing choice for bioproduction, especially in processes designed for the synthesis of reduced chemicals. *Pseudomonas* species display a unique combination of genetic and metabolic architectures when growing on glycerol as the main carbon substrate, in particular, *P. putida* and *P. aeruginosa*, where the issue has been examined to some extent. As indicated in the first part of this article, multi-omic strategies have strongly helped to elucidate the regulatory networks that rule glycerol utilization in *P. putida* KT2440 (including stochastic activation of genes encoding key enzymes needed for glycerol processing). *In silico*-guided metabolic engineering strategies have also been implemented to increase the production of PHAs from this substrate. Admittedly, the full potential of glycerol as a biotechnological substrate for *Pseudomonas* has not been fully realized yet, but promising avenues can be envisioned in the near future – including novel strategies merging synthetic biology designs and laboratory evolution of engineered strains (Nerholm, 2019). First, once regulatory constraints for
expression are overcome (e.g. by eliminating the GlpR repressor, as discussed above), core metabolic reactions linked to glycerol can be manipulated to foster synthesis of value-added C3 compounds. For example, the connection of glycerol to biomass formation could be severed, and enzymatic sub-networks could be set up for generating molecules of biotechnological interest, e.g. DHAP and derivatives thereof in resting cells [or, in any case, uncoupled from growth (Durante-Rodriguez et al., 2018; Volke et al., 2019)]. Along the same lines, glycerol metabolism could be refracted to reduce carbon loss as CO2, while either concomitantly or separately, adjusting the redox balance to provide a better intracellular environment for hosting transformations of interest on other substrates. Furthermore, several metabolic routes could be re-wired for fuelling the EDEMP cycle bottom-up by means of a synthetic C3 neogenesis, empowering NADPH overproduction from glycerol processing, particularly useful for biosynthesis of reduced bioproducts. To this end, genetic editing of the Pseudomonas metabolism will benefit from systems biology approaches for simulating and predicting the effects of given mutations on specific carbon fluxes and pathways (Cho and Palsson, 2009; Gray et al., 2015; Galardini et al., 2017).

A second, considerable challenge is the further adaptation to meet the composition of industrial-grade crude glycerol from biodiesel production. Typically, the glycerol stream has a polyol content in the range of 30-65% (wt/ vol), with the rest of the stream being CH3OH, fatty acid methyl esters, free fatty acids and glycerides together with ashes (Hu et al., 2012). The waste also has a high pH due to the residual KOH or NaOH carried on from upstream transesterification of oils and fats that generate biodiesel. Industrial-grade glycerol, often available as a non-homogeneous oily mixture, is obviously quite different to what one can have in the controlled and pure-substrate conditions of a shaken-flask cultivation in the laboratory. While pre-treatment (i.e. purification) may help improving the physical characteristics of this carbon source, bacteria have to ultimately face a mixture of compounds – some of them toxic and others not easily metabolizable. This offers again an opportunity to genetically knock in heterologous traits for the whole-cell catalyst to endure the stressful conditions imposed by the use of crude glycerol. The issue here includes both endurance to the toxic effect of the non-glycerol compounds of the mixture and the introduction of additional pathways for degrading or even growing on the additional carbon sources present in the medium. Some partial successes using industrial glycerol waste in bioproduction have been reported (see Table 1), yet the room for improvement in this field is still considerable.

Glycerol-based bioprocesses have to be run in bioreactors, with a very large liquid-to-biomass ratio and sterile culture media that, after the operation takes place, need to be processed for purification of the molecules of interest. This scenario makes the production of such compounds costly and only appealing when the price tag of the thereby-generated chemical is sufficiently high. The third avenue for improving glycerol valorization is therefore (re)designing the industrial engineering part of the bioprocesses better, and easing the downstream operations for reducing fermentation costs. This challenge not only applied to fermentations using this particular substrate, but to Microbial Biotechnology as a whole (de Lorenzo and Couto, 2019). Yet, even marginal improvement in bioprocess performance can make a considerable difference in the choice of substrates for feeding industrial-scale production. In each of these fronts, synthetic biology and metabolic engineering are bound to contribute to the overarching goals of sustainable production from renewable resources, zero waste, and circular management of feedstocks and products, in the frame of the so-called 4th Industrial Revolution (Schwab, 2017).

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Conflicts of interest

None declared.

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Glycerol as a substrate for Pseudomonas


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