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Refactoring the Embden–Meyerhof–Parnas Pathway as a Whole of Portable GlucoBricks for Implantation of Glycolytic Modules in Gram-Negative Bacteria

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Supporting Information

ABSTRACT: The Embden–Meyerhof–Parnas (EMP) pathway is generally considered to be the biochemical standard for glucose catabolism. Alas, its native genomic organization and the control of gene expression in Escherichia coli are both very intricate, which limits the portability of the EMP pathway to other biotechnologically important bacterial hosts that lack the route. In this work, the genes encoding all the enzymes of the linear EMP route have been individually recruited from the genome of E. coli K-12, edited in silico to remove their endogenous regulatory signals, and synthesized de novo following a standard (GlucoBrick) that enables their grouping in the form of functional modules at the user’s will. After verifying their activity in several glycolytic mutants of E. coli, the versatility of these GlucoBricks was demonstrated in quantitative physiology tests and biochemical assays carried out in Pseudomonas putida KT2440 and P. aeruginosa PAO1 as the heterologous hosts. Specific configurations of GlucoBricks were also adopted to streamline the downward circulation of carbon from hexoses to pyruvate in E. coli recombinants, thereby resulting in a 3-fold increase of poly(3-hydroxybutyrate) synthesis from glucose. Refactoring whole metabolic blocks in the fashion described in this work thus eases the engineering of biochemical processes where the optimization of carbon traffic is facilitated by the operation of the EMP pathway—which yields more ATP than other glycolytic routes such as the Entner–Doudoroff pathway.

KEYWORDS: Escherichia coli, Pseudomonas putida, standardization, glycolysis, metabolic engineering, PHB

The past few years have witnessed an increase in the number of microorganisms that can be metabolically engineered within the conceptual frame of Systems Biology and the molecular tools of contemporary Synthetic Biology. Longstanding platforms (e.g., Escherichia coli, Bacillus, Cornubacteria, and yeast) are increasingly accompanied by others (e.g., Pseudomonas species) that, due to their indigenous endurance to environmental stresses, have an improved ability to execute harsh biochemical transformations. Whether well-established or emerging, such platforms are empowered by a complex central carbon metabolic network where heterologous pathways must nest in any given host. Despite this fact, the majority of ongoing Metabolic Engineering efforts are preoccupied with what one could call peripheral aspects, e.g., the elimination of competing endogenous pathways, the fine-tuning of gene expression and substrate transport, and the rerouting of small molecules at given nodes of the network. In contrast, the biochemical core that fuels the microbial cell factory is generally taken for granted and hardly ever touched; i.e., few efforts have tried to reshape central enzymatic processes in its entirety. Relevant examples of this sort include auxotrophic CO2 fixation and CH3OH assimilation by engineered strains of E. coli. Such efforts suffer from the difficulties of re-engineering some of the most extremely interconnected and fine-tuned components of any biological system as central metabolism is.

Against this background, we wondered whether the most archetypal metabolic pathway (i.e., a linear glycolysis) could be reshaped in a way that, while maintaining its biochemical role and identity, could be freed of its native regulatory complexity and made portable either as a whole or as a functional subset of portable elements for specific Metabolic Engineering needs.

In its simplest definition, glycolysis is the metabolic breakdown of glucose into pyruvate (Pyr). Thought to have originated from simple chemical constraints in a prebiotic environment, glycolysis is one of the most widespread and conserved metabolic blocks in nature. Several biochemical sequences lead to the formation of Pyr from hexoses. The most studied example is the Embden–Meyerhof–Parnas (EMP) pathway, composed by the sequential activity of ten individual enzymes. The first five enzymes are involved in the so-called preparatory phase, which uses ATP to convert hexoses into trioses phosphate (i.e., glucose → glyceraldehyde-3-P (GA3P)). The pay-off phase comprises the second half of the EMP enzymes, and it yields 2 NADH molecules and 2 ATP molecules per each processed glucose molecule by converting...
trioses phosphate into Pyr \([i.e., \text{GA3P} \rightarrow \text{Pyr}]\). Therefore, the overall stoichiometry of the EMP pathway is glucose + 2NAD\(^+\) + 2ADP + 2Pi \rightarrow 2Pyr + 2NADH + 2H\(^+\) + 2ATP. Another glycolytic route is the Entner–Doudoroff (ED) pathway, widely distributed in prokaryotes (even more so than the EMP route). The overall stoichiometry of the ED pathway is glucose + NAD\(^+\) + NADP\(^+\) + ADP + Pi \rightarrow 2Pyr + NADH + NADPH + 2H\(^+\) + ATP. Although this biochemical sequence yields half the amount of ATP as compared to that of the EMP pathway,\(^{16}\) the ED route has been demonstrated to be a key source of reducing power in many environmental microorganisms.\(^{17,18}\) Because of a lifestyle of constant exposure to physicochemical insults often found in natural scenarios,\(^{19}\) such bacteria have evolutionarily favored the formation of reducing power\(^{20}\) to counteract oxidative stress.\(^{21}\) This is in contrast with the generation of ATP and the sheer buildup of biomass that appears to be the main metabolic driving force in Enterobacteria. Besides these highly conserved biochemical sequences, variants of carbohydrate breakdown processes are also present on other prokaryotes. Archaea, for instance, are characterized by the presence of unique, modified versions of the EMP and ED pathways which often include nonphosphorylating enzymes.\(^{22,23}\)

The research below was prompted by our ongoing efforts to establish the soil-dweller microorganism \textit{Pseudomonas putida} as a platform for hosting strong redox reactions.\(^{24–27}\) This bacterium lacks a 6-phosphofructo-1-kinase (Pfk) activity, and therefore the EMP pathway is not functional. The mere addition of Pfk to the biochemical network of \textit{P. putida} was not only insufficient to activate an EMP-based hexas catabolism, but it also resulted in deleterious effects on growth and limited resistance to oxidative stress.\(^{17}\) Thus, the channeling of glucose toward Pyr in \textit{P. putida}, while delivering enough NAD(P)H and yielding the highest possible amount of ATP, demands a multitiered approach involving more glycolytic genes or specific combinations thereof. To this end, in our present contribution we describe the reformattng of the entire EMP route of \textit{E. coli} in a layout that we have called GlucoBricks, that allows complete or partial implantation of the glycolytic route in a variety of Gram-negative bacteria including (but not limited to) \textit{P. putida}. This work finds inspiration in the attempts to decompress the regulatory density of the \textit{E. coli} T7 bacteriophage\(^{28}\) and the deconstruction/reconstruction of the whole \textit{Nt}-fixation system of \textit{Klebsiella oxytoca}\(^{29}\) in an easy-to-manipulate setup. This time, however, the objective was the implantation of central metabolic functions in different Gram-negative hosts by means of portable, standardized modules assembled in promiscuous plasmids belonging to the SEVA (\textit{Standard European Vector Architecture}) collection.\(^{30}\) The data discussed below not only demonstrates the versatility of the thereby reported device in various bacterial hosts, but it also expands the toolbox for deep engineering of central biochemical tasks in a microbial cell factory.

**RESULTS AND DISCUSSION**

**Functional Elements, Layout, and Design of Glycolytic Modules I and II.** The starting point of this research was to design a streamlined set of genes to aid implanting (or increasing) glycolytic capacities in the microbial cell. To this end, we first pinpointed each of the ten enzymes carrying out the complete transformation of glucose into Pyr through the EMP pathway in the extant metabolic network of \textit{E. coli}. In its naturally occurring configuration the corresponding genes are either scattered throughout the genome or arranged in two operons (\textit{e.g., \textit{fbaA} and \textit{pgk}}) under the transcriptional control of at least five regulators (Cra, SoxS, Crp, Fur, and FnrS) as well as a number of post-transcriptional control devices.\(^{31}\) Our first task was to exclusively capture the enzymatic complement of the system while releasing the corresponding genes (either separately or as a whole) from any host-specific regulatory
Figure 2. Genetic architecture of the GlucoBrick platform. (a) Physical map of Modules I and II, indicating restriction enzymes bracketing individual glycolytic genes. The enzyme targets are colored in the sequence of the multiple cloning site of all the plasmids belonging to the Standard European Architecture Vector to identify the DNA block they belong to (i.e., blue, Module I; and red, Module II). Other restriction targets that could be used to add different regulatory or structural elements and thereby expand the usability of this platform are shown in gray. The abbreviations used in this outline are G3P, glyceraldehyde-3-P; and Pyr, pyruvate. (b) Restriction analysis of Module I and II. Plasmids pS224-GBI (upper panel) and pS224-GBII (lower panel) were digested with the appropriate enzymes as indicated and the products were separated by electrophoresis in a 0.7% (w/v) agarose gel. Plasmid pS224 was digested with AvrII-BamHI (i, releases the whole Module I segment); AvrII-EcoRI (ii, releases glk); EcoRI-SacI (iii, releases pgI); SacI-KpnI (iv, releases pfkB); KpnI-Smal (v, releases fbaA); and Smal-BamHI (vi, releases pfkA). Plasmid pS224-GBI was digested with BamHI-HindIII (i, releases the whole Module II segment); BamHI-XbaI (ii, releases gapA); XbaI-SalI (iii, releases pgK); SalI-PstI (iv, releases gpmA); PstI-SphI (v, releases eno); and SphI-HindIII (vi, releases pykF).
when the corresponding plasmids were introduced in glycolytic
E. coli mutants lacking either single or several combined
activities of the EMP pathway (Table 1). E. coli BW25113, a
wild-type K-12 strain, was used as a positive control in all
these growth experiments, in which the final cell density and
the specific growth rate was recorded for each strain in cultures
containing both Km and IPTG. First, the initial steps in glucose
processing were targeted at the level of Glk (i.e., glucokinase)
and PtsI [i.e., the EI component of the phosphoenolpyruvate
(PEP):carbohydrate phosphotransferase (PTS) system]. No
glucose phosphorylation is possible in such mutant as Glk and
the PTS-dependent transport of the hexose coupled to
phosphorylation are both blocked.38 Note, however, that
glucose transport should not be significantly affected, since
alternative transporters (e.g., the low-affinity galactose:H+ GalP
symporter and the ATP-dependent MglABC system) internalize
the nonphosphorylated hexose when the PTS system is not
active.39 Accordingly, the Δglk ΔptsI strain grew in M9GCM
semisynthetic medium (formulated in such a way that all the
glycolytic mutants tested could grow, albeit some of them did
so poorly) but not in M9 minimal medium with glucose as the
only carbon source. The presence of Module I, however,
restored the growth of the double mutant on the hexose back
to the levels observed in the semisynthetic medium. When
the reaction catalyzed by Pfk (which mediates the glycolytic
formation of fructose-1,6-P$_2$ from fructose-6-P) was eliminated
by deleting both pfkA and pfkB, the growth of the resulting
strain was severely compromised even in M9GCM medium—
and no growth at all was observed in minimal medium with
glucose. The Pfk activity brought about by Module I was,
however, enough to restore this growth deficiency in both
culture media, and it also lead to a 2.3-fold increase in the
specific growth rate of the recombinant in M9GCM medium.

Another critical step of the EMP pathways is TpiA (i.e., triose
phosphate isomerase), which plays a central role not only in
downward glycolysis but also in gluconeogenesis.40 Expectedly,
the growth of a ΔtpiA mutant was impaired among all the
culture conditions tested, probably because of the buildup of
methylglyoxal as a toxic intermediate.41 Expression of tpiA
within Module I alleviated this metabolic situation, even
restoring the growth of the mutant from glucose and
significantly enhancing it in the semisynthetic medium.

The capability of Module II to mediate EMP activities in the
pay-off phase was analyzed in another set of E. coli glycolytic
mutants. The conversion of GA3P into PEP (and, con-
sequently, Pyr) was targeted to test Module II. Note that the
mere elimination of GA3P dehydrogenase in E. coli (repre-
sented by GapA, and possibly the GapB isozyme42) does not
block Pyr formation, as this metabolite could be also produced
by the PTS system from PEP. Most of the PEP pool comes
from the EMP pathway, yet anaplerotic reactions could also
generate this intermediate. Considering this complex metabolic
scenario (and in order to ensure that there is no Pyr
formation), a triple E. coli mutant was constructed by
eliminating gapA (encoding a NAD$^+$-dependent GA3P
dehydrogenase, and also the major GA3P dehydrogenase
prevalent in Enterobacteria), epd (also known as gapB,
encoding a NAD$^+$-erythrose-4-P dehydrogenase), and also
ptsI. Glucose-dependent growth was completely abolished in
this mutant, and M9GCM medium cultures only attained a
modest cell density. Once again, the presence of Module II
enhanced or even restored the growth of the triple mutant by
means of the GapA activity encoded therein. Interestingly, the
specific growth rate of the recombinant cells in semisynthetic
medium increased >6-fold when Module II was transformed in
the ΔgapA Δepd ΔptsI mutant—the highest among the
conditions and strains analyzed in this study. Finally, the
ΔpfkB and Δeno mutants had a similar qualitative behavior: they
grew very poorly in semisynthetic medium and could not grow
at all in M9 minimal medium with glucose. The corresponding

Table 1. Functional Validation of Module I and II in Glycolytic Mutants of Escherichia coli BW25113

<table>
<thead>
<tr>
<th>E. coli strain$^a$</th>
<th>plasmid$^b$</th>
<th>M9GCM semisynthetic medium</th>
<th>M9 minimal medium +20 mM glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CDW (g L$^{-1}$)</td>
<td>growth coefficient</td>
</tr>
<tr>
<td>BW25113 (wild-type strain)</td>
<td>None</td>
<td>2.7 ± 0.3</td>
<td>–</td>
</tr>
<tr>
<td>Δglk ΔptsI</td>
<td>pSEVA224</td>
<td>1.1 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>pS224-GBI</td>
<td>1.3 ± 0.3</td>
<td>–</td>
</tr>
<tr>
<td>ΔpfkB</td>
<td>pSEVA224</td>
<td>0.4 ± 0.1</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>pS224-GBI</td>
<td>1.4 ± 0.2</td>
<td>–</td>
</tr>
<tr>
<td>ΔtpiA</td>
<td>pSEVA224</td>
<td>0.6 ± 0.1</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>pS224-GBI</td>
<td>1.7 ± 0.3</td>
<td>–</td>
</tr>
<tr>
<td>ΔgapA Δpse</td>
<td>pSEVA224</td>
<td>0.5 ± 0.1</td>
<td>6.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>pS224-GBI</td>
<td>1.2 ± 0.1</td>
<td>–</td>
</tr>
<tr>
<td>Δpgk</td>
<td>pSEVA224</td>
<td>0.3 ± 0.1</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>pS224-GBI</td>
<td>1.4 ± 0.5</td>
<td>–</td>
</tr>
<tr>
<td>Δeno</td>
<td>pSEVA224</td>
<td>0.8 ± 0.2</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>pS224-GBI</td>
<td>1.5 ± 0.2</td>
<td>–</td>
</tr>
</tbody>
</table>

*The detailed genotype of the strains is given in Table S1 in the Supporting Information. $^b$Plasmid pSEVA224 was used as the control vector; plasmids pS224-GBI and pS224-GBI were pSEVA224 derivatives carrying either Module I or II, respectively. IPTG was added to all cultures at 1 mM at the onset of the cultivation. M9GCM semisynthetic medium contains the same salts as M9 minimal medium, casein hydrolyzate, glycerol, and sodium malate. CDW, cell dry weight after 48 h of aerobic incubation at 37 °C. N.G., no growth (defined as a change in the CDW < 0.05 g L$^{-1}$). The growth coefficient is the ratio between the specific growth rate of a strain carrying either Module I or II and the specific growth rate of the same strain carrying only pSEVA224; in the cases in which the mutant did not grow (and hence, the growth coefficient could not be calculated) are indicated as G.R., growth restored. Results represent the mean value ± standard deviation from triplicate measurements in at least two independent experiments. All the differences in growth coefficients for the strains grown in M9GCM semisynthetic medium were significant (P < 0.05, as evaluated by means of the Student’s t test) in the pairwise comparison of a given recombinant to the control strain carrying the empty pSEVA224 vector.
Figure 3. Characterization of physiological parameters in recombinant *Pseudomonas putida* and *P. aeruginosa* strains carrying Module I. (a) Schematic representation of central carbon metabolism in *Pseudomonas* species. Glucose catabolism occurs mainly through the activity of the Entner–Doudoroff (ED) pathway, but part of the trioses-P thereby generated are recycled back to hexoses-P by means of the EDEMP cycle, that also encompasses activities from the Embden–Meyerhof–Parnas (EMP) and the pentose phosphate (PP) pathways. Note that a set of peripheral reactions can also oxidize glucose to gluconate and/or 2-ketogluconate (2KG) before any phosphorylation of the intermediates occurs. Each metabolic block is indicated with a different color along with the relevant enzymes catalyzing each step, and the EDEMP cycle is shaded in blue in this diagram. Note that the 6-phosphofructo-1-kinase activity, missing in most *Pseudomonas* species, is highlighted with a dashed gray arrow. The abbreviations used for the metabolic intermediates are as indicated in the legend to Figure 1; other abbreviations are as follows: 6PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; acetyl-CoA, acetyl-coenzyme A; 2KG, 2-ketogluconate; and 2K6PG, 2-keto-6-phosphogluconate. (b) Glucose consumption profile and (c) growth curves of *P. putida* KT2440, its Δglk derivative, and *P. aeruginosa* PAO1, carrying either the control vector (pSEVA224) or pS224-GBI (Module I). Glucose consumption is reported as the mean value ± standard deviation from duplicate measurements in at least three independent experiments. CDW, cell dry weight. Significant differences (*P* < 0.05, as evaluated by means of the Student’s *t* test) in the pairwise comparison of a given recombinant to the control strain, carrying the empty pSEVA224 vector, are indicated by an asterisk. In the growth curves, each data point represents the mean value of the optical density measured at 600 nm (OD600) of quadruplicate measurements from at least three independent experiments. The specific growth rates were calculated from these data during exponential growth, and the inset shows the mean values ± standard deviations for each strain.

activities encoded in Module II sufficed to restore the growth of the corresponding recombinants on the hексозе, while the specific growth rate in M9GCM medium increased by ca. 3-fold in either case (Table 1). Note that, besides the functional complementation of individual enzyme activities missing in the single or multiple mutant strains studied herein, the GlucoBrick blocks can also amplify glycolytic activities that are already in place in *E. coli*, thus boosting the channeling of hexoses through the linear EMP pathway. In all the cases studied here, the final cell densities attained by the recombinants carrying GlucoBricks in M9 minimal medium with glucose was comparable to that of wild-type *E. coli* BW25113.

In order to evaluate if these growth phenotypes correlate with higher specific activities of some of the enzymes involved in glucose processing, some key steps in the EMP pathway were evaluated in cell-free extracts in *vitro*. The activity of Glk in the glucose-grown Δglk ΔptsI mutant expressing Module I was 1,370 ± 120 nmol min⁻¹ mg protein⁻¹ upon induction of gene expression with IPTG, representing a ca. 23- and 10-fold increase as compared with the same strain carrying the empty vector and wild-type BW25113 carrying the empty vector, respectively (note that some residual Glk activity was observed in the knockout strain, possibly arising from some other nonspecific hexose kinases⁴⁻⁵). Similarly, introduction and induction of the genes in Module I in the ΔpfkAΔpfkB strain resulted in 10-fold increase in the Pfk activity (1,080 ± 70 nmol min⁻¹ mg protein⁻¹) as compared with the same strain carrying the empty pSEVA224 vector. In contrast, the Pfk activity in *E. coli* BW25113 was 751 ± 36 nmol min⁻¹ mg protein⁻¹. The residual in *vitro* phosphofructokinase activity observed in the *E. coli* ΔpfkA ΔpfkB mutant (<100 nmol min⁻¹ mg protein⁻¹) could arise from a side activity of an enzyme belonging to the PfkB family of phosphosugar kinases, which form a subset of the large ribokinase superfamily.⁴⁴ Possibly, ribokinase (or another enzyme belonging to this family) phosphorylates fructose-6-P to fructose-1,6-P₂ in *vitro*, which in turn may be due to the dilution of an allosteric inhibitor in the assay as previously described by Chin and Cirino.⁴⁵ Together, taken these results indicate that the efficiency of either Glk or Pfk in *E. coli* can be appropriately complemented by means of the activities borne by the GlucoBrick platform. The evidence gathered so far highlight the versatility of the GlucoBrick system in bestowing or even enhancing glycolytic activities in *E. coli* mutants—the next relevant question being whether this tool can also be used in other, unrelated bacterial species.

**The Activities Encoded by GlucoBricks Are Instrumental for Engineering Glycolysis in Two Pseudomonas Species.** Pseudomonads are a wide group of aerobic Gram-negative γ-proteobacteria characterized by their remarkable metabolic versatility and ubiquitous presence in many environmental niches.⁴⁶–⁴⁸ *P. putida* and *P. aeruginosa* are representa-
tive members of the genus, which prevalently use the ED pathway for hexoses breakdown.\textsuperscript{25,49} P. putida KT2440, a current platform for Synthetic Biology and Metabolic Engineering,\textsuperscript{21} uses a cyclic combination of enzymes of the EMP pathway, the ED pathway, and the pentose phosphate pathway to catabolize glucose \textit{via} a metabolic array termed as \textit{EDEMP cycle}\textsuperscript{26} (Figure 3a)—probably operative in other members of the \textit{Pseudomonas} group as well, such as \textit{P. aeruginosa}. The intrinsic metabolic complexity of glycolysis in these bacteria makes the targeted Metabolic Engineering of primary metabolism particularly difficult, and we thus decided to adopt both \textit{P. putida} KT2440 and \textit{P. aeruginosa} PAO1 as the heterologous hosts for testing the GlucoBrick platform by assessing physiological and growth parameters as well as the \textit{in vitro} activity of key glycolytic enzymes.

Since the expression of the glycolytic modules is expected to boost the activity of the extant EMP pathway enzymes in \textit{Pseudomonas} and to provide, at the same time, the missing step catalyzed by Pfk, we evaluated the overall glucose consumption in recombinant \textit{P. putida} and \textit{P. aeruginosa} strains carrying either an empty pSEVA224 vector or the pS224-GBI plasmid, bearing Module I (Figure 3b). In both cases, the overall rate of glucose consumption was boosted by expressing the genes of Module I (\textit{i.e.}, 2.5- and 1.5-fold in \textit{P. putida} KT2440 and \textit{P. aeruginosa} PAO1, respectively, as compared to the strains bearing the pSEVA224 vector). Interestingly, no major growth deficiencies (both in terms of final biomass densities and specific growth rates) were observed in the strains carrying Module I (Figure 3c). \textit{P. aeruginosa} PAO1 had a slight reduction in the specific growth rate when expressing Module I, but the difference in this parameter as compared to that in the same strain carrying an empty vector was not significant. In any case, this result is somewhat in contrast with the observed deleterious effect of separately overexpressing \textit{pfkA} from \textit{E. coli} in \textit{P. putida} KT2440.\textsuperscript{1,7} It is plausible that the differences arise from expressing the kinase gene alone versus introducing the whole upper metabolic block from \textit{E. coli} in \textit{P. putida}—thereby enabling circulation of carbon from glucose down to G3P by connecting the corresponding reactions. The consequences of blocking this situation, broadly known as \textit{metabolic channeling}, have been recently described in a \textit{Δpfk} mutant of \textit{E. coli}.
\textsuperscript{59} The authors indicate that intermediates of the EMP pathway are passed from one enzyme to the next one without equilibration within the cellular medium. As such, all the enzymes in the biochemical sequence are needed to reach an efficient traffic through the whole pathway—a finding that helps explaining why the concerted expression of all the EMP genes in \textit{P. putida} KT2440 does not impact the cell physiology significantly.

If the hypothesis of metabolic channeling advanced above holds true, one might expect that an increase in the activities of the whole EMP pathway is a \textit{conditio sine qua non} for activating a linear glycolysis in \textit{Pseudomonas} species. We therefore decided to evaluate if the glycolytic activities implanted could actually account for a functional EMP pathway by evaluating the \textit{in vitro} activities of enzymes of the preparatory phase of this pathway in different \textit{Pseudomonas} recombinants.

The activity of Glk was first assayed in strains KT2440 and PAO1 carrying pS224-GBI (Module I) and growing on glucose as the sole carbon source, and added with Km and IPTG as needed (Figure 4a). Unlike \textit{E. coli}, \textit{Pseudomonas} species do not have a devoted PTS system for glucose uptake, and direct phosphorylation of the hexose is catalyzed by Glk \textit{(i.e., PP_1011 in \textit{P. putida} and PA_3193 in \textit{P. aeruginosa})} in the cytoplasm. The native Glk activity in both \textit{Pseudomonas} species ranged from \textit{ca.} 89 to 184 nmol min\textsuperscript{−1} mg protein\textsuperscript{−1} in \textit{P. aeruginosa} PAO1 and \textit{P. putida} KT2440, respectively, and this kinase activity was boosted by expression of \textit{E. coli} glk from plasmid pS224-GBI \textit{(i.e., a 1.4-fold increase in strain KT2440 and a remarkable 6.2-fold increase in strain PAO1)}. A \textit{P. putida} \textit{Δglk} strain was also constructed, in which the \textit{in vitro} Glk activity resulted negligible. Transformation of this glk mutant strain with plasmid pS224-GBI and induction of gene expression by IPTG addition augmented the kinase activity up to 45 nmol min\textsuperscript{−1} mg protein\textsuperscript{−1} \textit{(i.e., a 100-fold increase in Glk activity)}.

As mentioned before, a Pfk activity is altogether missing in several \textit{Pseudomonas} species, strains KT2440 and PAO1 being prime examples of the absence of such glycolytic step. Thus, the introduction of Module I in these strains would fill the gap between fructose-6-P and fructose-1,6-P\textsubscript{2}, by grafting the PfkA activity from \textit{E. coli}. \textit{This in vitro} kinase activity was analyzed on both \textit{Pseudomonas} species transformed with plasmid pSEVA224-GBI and grown in M9 minimal medium containing

Figure 4. Biochemical characterization of native and implanted enzyme activities in \textit{Pseudomonas} species. (a) \textit{In vitro} quantification of the specific (Sp) glucokinase (Glk) activity, which phosphorylates glucose to glucose-6-P (G6P) in wild-type (WT) \textit{P. putida} KT2440 and its \textit{Δglk} derivative (left panel), and WT \textit{P. aeruginosa} PAO1 (right panel) carrying either the empty pSEVA224 vector or Module I. (b) \textit{In vitro} quantification of the specific (Sp) 6-phosphofructo-1-kinase (PfkA) activity, which converts fructose-6-P (F6P) into fructose-1,6-P\textsubscript{2} (FBP) in WT \textit{P. putida} KT2440 and its \textit{Δglk} derivative (left panel), and WT \textit{P. aeruginosa} PAO1 (right panel) carrying either the empty pSEVA224 vector or Module I. (c) \textit{In vitro} quantification of the specific (Sp) aldolase, phosphoglucoisomerase, and triose phosphate isomerase in \textit{P. putida} KT2440 carrying either the empty pSEVA224 vector or Module I. These three activities, combined with Glk and PfkA, constitute the preparatory phase of the Embden–Meyerhof–Parnas pathway \textit{(i.e., from glucose to glyceraldehyde-3-P)}. All the strains tested were grown on M9 minimal medium added with glucose at 20 mM and cells were harvested in midexponential phase for these \textit{in vitro} enzymatic assays. Each bar represents the mean value of the corresponding enzymatic activity ± standard deviation of quadruplicate measurements from at least two independent experiments. Significant differences \textit{(P < 0.05, as evaluated by means of the Student’s t test)} in the pairwise comparison of a given recombinant to the control strain, carrying the empty pSEVA224 vector, are indicated by an asterisk.
glucose, Km, and IPTG (Figure 4b). Expectedly, almost no Pfk activity was detected neither in P. putida nor in P. aeruginosa. Expression of the genes borne by plasmid pS224-GBI resulted in increased levels of Pfk activity, which were similarly high in both P. putida KT2440 and P. aeruginosa PA01 (i.e., ca. 272 and 550 nmol min⁻¹ mg protein⁻¹, respectively). The change in the kinase activity brought about by heterologous expression of pfkA in P. putida Δglk was somewhat low, but it still represents a 23-fold increase as compared to the control strain transformed with an empty vector. The differences in the activities detected in the wild-type strain and its Δglk derivative likely arise from alterations in the intracellular metabolite pools, which may determine a different pattern of regulation on the PfkA enzyme.

The three remaining activities within the preparatory phase (i.e., aldolase, phosphoglucone isomerase, and triose phosphate isomerase) of the EMP pathway were also evaluated in vitro in glucose-grown P. putida KT2440 carrying either pSEVA224 or pS224-GBI (Figure 4c). All three enzyme activities had a significant increase in the recombinant carrying Module I as compared to the same strain transformed with the empty pSEVA224 vector. In particular, the total aldolase and triose phosphate isomerase activities had a 3- and 5-fold increase, respectively, in P. putida KT2440 expressing Module I, whereas the activity of triose phosphate isomerase augmented a surprising 43-fold. Taken together, these in vitro results indicate that all the enzymes within the preparatory phase of the EMP pathway are active upon introduction of Module I in P. putida, thereby accounting for a complete linear glycolysis. Yet, if this is the case, one would also expect an impact of these manipulations on the intracellular metabolome in the engineered strain—an issue that was investigated as disclosed below.

**Impact of the Glucobrick Platform in the Intracellular Metabolome of P. putida KT2440.** The next step was to evaluate the intracellular concentration of some critical metabolic intermediates in P. putida KT2440 carrying either pSEVA224 or pS224-GBI. The first intermediate after glucose phosphorylation (i.e., glucose-6-P), and the end product of the preparatory phase of the EMP pathway (i.e., GA3P) were targeted and their concentration was measured by means of liquid chromatography coupled to mass spectrometry (Table 2). Both glycolytic building blocks had an increased abundance in the strain expressing the genes of Module I (i.e., 1.7-fold in the case of the hexose-P and 3.2-fold in the case of the triose-P), a further experimental indication that the EMP pathway is active in this recombinant strain. Moreover, if glucose gets channeled into a linear EMP route instead of the native EDEMP cycle, a reduction in the NADPH availability (that would be otherwise generated through the activity of Zwf, see Figure 3a) is to be expected. The direct measurement of this redox cofactor confirms that is actually the case: the P. putida KT2440 derivative expressing Module I showed a 26% reduction in the intracellular content of NADPH (Table 2). Taken together, these targeted metabolomic determinations (along with the measurement of EMP enzyme activities) indicate that glucose is channeled into a linear glycolysis of sorts in P. putida KT2440 when expressing the genes within Module I.

The results of the two preceding sections highlight the versatility of the Glucobrick platform to boost existing glycolytic activities or to introduce new metabolic steps that are alien to the biochemical network of Pseudomonas, thereby facilitating the engineering of primary metabolism not only in E. coli but also in unrelated bacterial species. Against this background, the next step was the functional evaluation of this Synthetic Biology tool in the context of the ongoing efforts aimed at manipulating the production of added-value metabolites.

**Enhancing Heterologous PHB Production by Boosting Glycolytic Activity in Recombinant E. coli.** Polyhydroxyalkanoates are a complex family of bacterial biopolymers.51–53 PHB is an isotactic polyester composed by 3-hydroxybutyrate units.54 The PHB synthesis pathway in *Cupriavidus necator* (formerly known as *Ralstonia eutropha*) comprises three enzymes (Figure 5a). PhaA, a 3-ketoacyl-CoA thiolase, condenses two acetyl-CoA moieties, yielding 3-acetoacetyl-CoA. This intermediate is the substrate for PhaB, a NADPH-dependent 3-3-acetoacetyl-CoA reductase (encoded by phaB1). In the final step of this biosynthetic pathway, (R)-(−)-3-hydroxybutyryl-CoA units are polymerized to PHB by PhaC, a PHA synthase (encoded by phaC1). The idea of a thermoplastic and biocompatible material which is also readily biodegraded by a number of bacteria has become very attractive in an era of increasing environmental concern and shortage of oil supply.55 A number of different recombinant *E. coli* strains have been constructed thus far by outsourcing structural and regulatory pha genes from several bacterial species,56 given that *E. coli* does not possess the metabolic machinery needed for the synthesis or the degradation of polyhydroxyalkanoates. A problem recursively encountered when attempting to improve the yield and productivity of biopolymer in recombinant *E. coli* strains is that the PHB biosynthetic pathway is nested in the core biochemical network of this bacterium,57 not only drawing intermediates in Glucose-Grown *C. necator* (formerly known as *Ralstonia eutropha*) into wild-type *E. coli* BW25113.58 Plasmids pSEVA224 (KmR, empty vector), pS438-GBI (SmR, carrying Module I), and/or pS224-GBII (KmR, carrying Module II) were introduced in this strain, and growth, polymer accumulation, and acetate excretion were evaluated in LB medium cultures added with glucose and the corresponding antibiotics and inducers after 24 h of aerobic growth (Table 3)}
or modules indicated (see Table 2 for further details). Cells were grown aerobically in LB medium added with glucose at $10 \text{ g L}^{-1}$. 6PG, 6-phosphogluconate; acetyl-CoA, acetyl-coenzyme A; OAA, as shown in the caption to Figure 1; other abbreviations are as follows: gluconeogenesis, green. Abbreviations of metabolic intermediates are Di

in the major fermentation pathway of molecule per monomer added. Note that acetyl-CoA can also be used merizes 3-HB-CoA monomers to PHB by releasing one CoA-SH to 3-acetoacetyl-CoA and the reduction of this intermediate to acetoacetyl-CoA reductase (PhaB1), and a PHB synthase (PhaC1). PHB accumulation depends on the sequential activity of a 3-ketoacyl-

standard deviation from duplicate measurements in at least three independent experiments. N.D., not determined.

plasmid can be found in Table S2 in the Supporting Information.

h. Each parameter is reported as the mean value from duplicate measurements in at least three independent experiences. P

Table 3. Growth Parameters and Polymer Synthesis of a PHB-Accumulating Escherichia coli BW25113 Strain Carrying Different Combinations of Glycolytic Genes

<table>
<thead>
<tr>
<th>plasmid</th>
<th>CDW</th>
<th>PHB (g L$^{-1}$)</th>
<th>$\mu$ (h$^{-1}$)</th>
<th>specific rate of acetate formation (mmol g CDW$^{-1}$ h$^{-1}$)</th>
<th>acetyl-coenzyme A content (mmol mg CDW$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSEVA224 (empty vector)</td>
<td>0.54 ± 0.06</td>
<td>0.21 ± 0.04</td>
<td>0.46 ± 0.03</td>
<td>3.3 ± 0.4</td>
<td>0.43 ± 0.09</td>
</tr>
<tr>
<td>pS438-GBI (Module I)</td>
<td>0.35 ± 0.02</td>
<td>0.43 ± 0.02</td>
<td>0.29 ± 0.03</td>
<td>1.1 ± 0.2</td>
<td>0.75 ± 0.03</td>
</tr>
<tr>
<td>pS224-GBI (Module II)</td>
<td>0.37 ± 0.01</td>
<td>0.61 ± 0.01</td>
<td>0.29 ± 0.01</td>
<td>1.8 ± 0.2</td>
<td>0.59 ± 0.04</td>
</tr>
<tr>
<td>pS438-GBI + pS224-GBI</td>
<td>0.11 ± 0.01</td>
<td>0.35 ± 0.01</td>
<td>0.21 ± 0.02</td>
<td>1.3 ± 0.1</td>
<td>N.D.</td>
</tr>
<tr>
<td>pS424-gapA</td>
<td>0.45 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>0.44 ± 0.01</td>
<td>2.8 ± 0.3</td>
<td>N.D.</td>
</tr>
<tr>
<td>pS224-GPl (glk-pfKA-gapA)</td>
<td>0.32 ± 0.01</td>
<td>0.72 ± 0.01</td>
<td>0.42 ± 0.02</td>
<td>1.5 ± 0.1</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

E. coli BW25113 carrying plasmid pActT41 (i.e., constitutively expressing the phaC1ABI gene cluster from C. necator) was transformed with the plasmids indicated, and grown aerobically in LB medium added with glucose at $10 \text{ g L}^{-1}$ for 24 h. Each parameter is reported as the mean value ± standard deviation from duplicate measurements in at least three independent experiments. N.D., not determined. The full description of each plasmid can be found in Table S2 in the Supporting Information. The residual cell dry weight (CDW$_r$) was calculated as the difference between the total CDW and the PHB concentration.
The residual CDW of either strain under PHB accumulation conditions was similar (Table 3), indicating that neither Module I nor II affected biomass formation. Expectedly, the simultaneous expression of all GlucoBricks from two independent plasmids further boosted polymer accumulation up to 72.5 ± 9.8% of the CDW—thus representing an increase of ca. 3-fold as compared to E. coli BW25113/pAeT74 + pSEVA224. Interestingly, the overexpression of the glycolytic modules did not lead to an increase in overflow metabolism since acetyl-CoA was rerouted into PHB accumulation rather than acetate formation. This result further illustrates how the availability of key precursors in central metabolism can be channeled into the formation of an added-value product while minimally affecting the physiology of the bacterial host.

In an attempt to determine which glycolytic steps were determinant in increasing PHB accumulation by these recombinants, two different SEVA plasmids were constructed, in which individual GlucoBricks belonging to either Module I or II are expressed under the transcriptional control of an LacI2/2/Tcr regulatory element. Plasmids pS424-gapA and pS224-GPG, carrying glk, pfkA, and gapA (Table S2 in the Supporting Information) were separately introduced into E. coli BW25113/pAeT41, and growth, glucose consumption, and PHB accumulation were tested under the same culture conditions indicated above. While an increase in the traffic through GA3P dehydrogenase (i.e., GapA) did not result in a significant increase of either glucose consumption or polymer synthesis, the expression of the glk:pfkA-gapA cluster from plasmid pS224-GPG significantly enhanced both parameters with respect to the control strain (Figure 5b and c). PHB accumulation in this recombinant peaked at 66.4 ± 3.7% of the CDW (although there was no differences in the actual PHB concentration, see Table 3). Yet, boosting the glycolytic traffic by means of Glk, Pfk and GapA alone was not enough to reach the highest accumulation of PHB, as observed in the strain bearing both Modules I and II. This result may in turn suggest that an appropriate channeling of metabolic intermediates from glucose all the way down to acetyl-CoA is only possible if an adequate balance of all the individual enzymatic activities involved is met. Such scenario also supports the notion that all the enzymatic activities of glucose all the way down to acetyl-CoA is only possible if an adequate balance of all the individual enzymatic activities involved is met. Then, tuning primary metabolism is in principle an effective strategy to foster availability of key carbon intermediates. Yet, the only attempts for, e.g., increase the glycolytic activities of E. coli have thus far focused on individual genes. For instance, Solomon et al. designed a recombinant E. coli improved for gluconate production by endowing it with different (and adjustable) levels of Glk activity. However, the biological activities of the synthetic EMP route presented here demonstrate the portability and efficiency of some block metabolic pathways (or subsets of them) formerly thought to be short of untouchable and/or difficult to handle as a multienzyme whole.

Besides validating the autonomy of the implanted EMP route, the data above show the utility of a flexible toolbox for boosting the efficiency of carbon consumption and distribution in a variety of cell factories of biotechnological interest. Some of the engineered modules were able to recover glucose-dependent growth of E. coli mutants (deficient in either individual or several glycolytic steps) to an extent beyond the wild-type situation. Increasing glycolytic activities in PHB-accumulating recombinant E. coli resulted in polymer contents (up to ca. 73% of the CDW) that rank among the highest reported in the literature for batch cultures using glucose as the carbon source. From a different perspective, and owing to the broad-host-range vectors and expression systems available, the enzymatic activities endowed by the platform could be promiscuously transferred to other species (e.g., Pseudomonas, as illustrated in the present study) or even to complete microbial communities (a development of the platform currently under development in our laboratory). Finally, the modular arrangement of the GlucoBricks and the possibility to assemble them as promoter-less gene arrays in SEVA-compatible transposon vectors allows exploration of the right level of expression that makes them more effective when knocked-in different heterologous hosts (which would also reduce any possible metabolic burden related to plasmid maintenance and/or addition of inducers). Further improvements are expected as long as the expression of the genes within each module is appropriately adjusted, and a minimal subset of glycolytic genes, promoting an efficient EMP-based catabolism, is identified. The specific impact of the GlucoBricks on the metabolic networks of different recipients is likely to differ depending on the bacterial species, an issue that can be addressed wherever necessary by measuring fluxes with 13C-labeled substrates. In sum, we believe that the GlucoBricks concept herein presented provide a valuable example of how rationally formatting biological constituents eases the engineering of new-to-nature properties with a reasonable degree of efficiency and predictability.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions. The bacterial strains and plasmids used in this study are listed in Table S1 and Table S2, respectively, in the Supporting Information. E. coli and P. aeruginosa were grown at 37 °C; P. putida cultures were incubated at 30 °C. For regular growth and for the propagation and construction of plasmids, E. coli strains CC118 and DH5α pir were cultured in LB medium. For physiology experiments and in order to obtain cell-free extracts for enzyme assays, bacterial cells were grown with rotary agitation at 170 r.p.m. in 250-ml Erlenmeyer flasks filled with 50 mL of M9 minimal medium, containing 6 g L−1 Na2HPO4, 3 g L−1 KH2PO4, 1.4 g L−1 (NH4)2SO4, 0.5 g L−1 NaCl, 0.2 g L−1 MgSO4 · 7H2O, and 2.5 mL L−1 of a trace elements solution. When culturing E. coli strains in minimal media, CaCl2 was added at 0.1 mM and vitamin B1 was added.
at 0.05% (w/v). Unless indicated otherwise, minimal medium cultures were added with glucose at 20 mM and IPTG at 1 mM as explained in the text. The growth of some glycolytic mutants of *E. coli* is known to be impaired even in rich LB medium,66 these mutant cultures were grown in M9GM semisynthetic medium. This medium contains the same salts as M9 minimal medium, but also 0.75% (w/v) amino acids from casein hydrolyzate (Becton-Dickinson Diagnostics Co., Sparks, MD, USA), 10 mM glucose, 20 mM glycerol, 15 mM sodium malate, and 0.05% (w/v) vitamin B1. In the case of *E. coli* ΔgapA Δpd and ΔgapA Δpd Δtpsi mutants (i.e., deficient in the epd-encoded erythrose-4-P dehydrogenase), pyridoxine hydrochloride was added to the culture medium at 5 μM.67 For the purpose of adapting the cells to growth on glucose from rich LB medium, preinocula, prepared with a few isolated colonies from LB medium plates, were grown overnight in 10 mL of M9 minimal medium with glucose or M9GM semisynthetic medium with the corresponding antibiotics in 100-mL Erlenmeyer flasks. Solid media used to streak cells contained 15 g L⁻¹ agar. The antibiotics employed for selection were added to the media when needed at the following final concentrations: ampicillin, 150 μg mL⁻¹ for *E. coli* strains or 500 μg mL⁻¹ for *P. putida* strains; and Km, 50 μg mL⁻¹ with the exception of *P. aeruginosa*, for which 300 μg mL⁻¹ Km was needed to select colonies after transformation of plasmids by electroporation. For the construction of *P. putida* mutants, 3- methylbenzoate (3-mBz) was used at 15 mM to induce the YxS-dependent Pm promoter. Experiments for PHB accumulation were carried out in LB medium added with glucose at 10 g L⁻¹ and the antibiotics and inducers described in the text (in these experiments, 3-mBz was used at 0.5 mM to induce the expression of the genes in Module I).

**DNA Manipulation and Sequencing, and Construction of Mutant Strains.** DNA manipulations followed routine laboratory techniques.65 Plasmid DNA was obtained with the QIaprep Spin Miniprep kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions. Oligonucleotides were ordered from Sigma-Aldrich Co. (St. Louis, MO, USA). Restriction and DNA modification enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Isolated colonies from fresh LB medium plates were used as the starting material in colony PCR amplifications for checking gene deletions or the presence of plasmids. PCR products were purified with the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany). Agarose gel visualization was carried out using a Molecular Imager VersaDoc apparatus (Bio-Rad Corp., Hercules, CA, USA). Electroporation of plasmid DNA in *P. putida* and *P. aeruginosa* was carried out as indicated by Choi et al.68 and in *E. coli* as described by Datsenko and Wanner.57 The accuracy of all the DNA constructs was confirmed by Sanger sequencing (Secugen SL, Madrid, Spain). A clean *P. putida* glk knockout mutant was obtained following the protocol described by Martinez-Garcia and de Lorenzo.69 The detailed protocol for the construction of this Δglk mutant is described in the Supporting Information. Mutations in glycolytic genes were accumulated in *E. coli* derivatives via sequential transduction of individual alleles with bacteriophage P136,70 using individual mutants from the KEIO collection14 as donors, followed by elimination of the antibiotic resistance marker using plasmid pCP20.2

**Design and Assembly of the GlucoBrick Modules.** DNA sequences of the glycolytic genes of *E. coli* strain K-12 substrain MG1655 were obtained from the web-based EcoCyc database collection (GenBank accession number U00962). The genes were assembled in two blocks, the first of them, termed GlucoBrick Module I, spanning glk (b2388), pgI (b4025), pKA (b3916), fbaA (b2925), and tpiA (b3919); and the second one, termed GlucoBrick Module II, comprising gapA (b1779), pgK (b2926), gpmA (b0755), eno (b2779), and pykF (b1676). Each gene was preceded by a synthetic RBS (5′-AGG AGG AAA AAC AT-3′), and the structural coding sequences were manually edited to erase targets for restriction enzymes present in SEVA plasmids (i.e., AvrII, EcoRI, SacI, KpnI, SmaI, BamHI, XbaI, SalI, PstI, SphI, and HindIII) while conserving the amino acid sequence of the encoded polypeptides. The two blocks were synthesized de novo by GeneCust Europe (GeneCust Laboratoire de Biotechnologie du Luxembourg S.A.; Dudelange, Luxembourg), and checked for accuracy by Sanger sequencing. The two DNA modules were subcloned in pSEVA vectors to obtain the plasmids indicated in Table S2 in the Supporting Information. Rapid screening of recombinants carrying Module I was carried out by colony PCR using oligonucleotides pKAf/bA-Check-F and pKAf/bA-Check-R (Table S3 in the Supporting Information). In the case of Module II, oligonucleotides gpmA-eno-Check-F and gpmA-eno-Check-R were used (Table S3 in the Supporting Information).

**Preparation of Cell-Free Extracts and In Vitro Enzymatic Assays.** Cell-free extracts of *E. coli*, *P. putida*, and *P. aeruginosa* were obtained by following published protocols.71–78 A detailed description of the procedure and the specific methods used for in vitro assays of Glk, Pfk, and GAP3 dehydrogenase are given in the Supporting Information. The limit of detection for all the enzymatic assays was consistently below 2–5 nmol min⁻¹ mg protein⁻¹.

**Analytical Determinations.** PHB quantification was carried out by flow cytometry after staining the cells with Nile Red as indicated by Martinez-Garcia et al.,32 and by means of gas chromatography as described elsewhere.79 Acetate concentration in culture supernatants of selected samples was determined by using a commercially available enzymatic kit essentially as indicated by Nikel et al.80 with the modifications described by Nikel and de Lorenzo.81 The intracellular concentration of acetyl-CoA was determined as described by Pfüger-Grau et al.,82 with the adjustments specified by Martinez-Garcia et al.44 An adapted protocol based on the glucose assay kit of Sigma-Aldrich Co. was used to quantify residual glucose in culture supernatants in 96-well microtiter plates (NunclonΔSurface; Nunc A/S, Roskilde, Denmark). The assay reagent was prepared as indicated in the technical bulletin; the final mix per well contained 80 μL of the assay reagent, 40 μL of the sample (diluted with water to approximately 20–80 μg glucose mL⁻¹), and 80 μL of 12 N H₂SO₄. The amount of the final pink-colored product (oxidized o-dianisidine) was quantified at 540 nm using a SpectraMax M2e plate reader (Molecular Devices, LLC., Sunnyvale, CA, USA). The supernatants for these determinations were obtained by centrifugation of 50 mL cultures (grown for 24 h) at 4000 r.p.m. for 15 min at 4 °C.

**Determination of Intracellular Metabolite Concentrations.** *P. putida* cultures carrying either the empty vector pSEVA224 or pS224-GBI were grown in M9 minimal medium containing glucose at 20 mM as explained above until they reached the midexponential phase (i.e., optical density measured at 600 nm of ca. 0.5), at which point the biomass corresponding to 0.5–0.6 mg of CDW was collected in duplicates by fast centrifugation (13 000 r.p.m., 30 s, −4 °C).
Bacterial pellets were immediately frozen by immersing the cell sediment in liquid N\textsubscript{2}. Samples were then extracted three times with 0.5 mL of 60% (v/v) ethanol buffered with 10 mM ammonium acetate (pH = 7.2) at 78 °C for 1 min. After each extraction step, the biomass was separated by centrifugation at 13 000 r.p.m. for 1 min. The three liquid extracts were pooled in a new tube and dried at 120 μL, and finally stored at −80 °C. Samples were resuspended in 20 μL of Milli-Q water and injected into a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA) with a Waters Acquity T3 column (150 mm × 2.1 mm × 1.8 μm, Waters Corp.) coupled to a Thermo TSQ Quantum Ultra triple quadrupole instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA) with electrospray ionization. The quantitative analysis of raw metabolomic data and the normalization procedure were conducted as explained by Nikel et al.\textsuperscript{26} and van der Werf et al.\textsuperscript{83}

### Statistical Analysis

All the experiments reported were independently repeated at least twice (as indicated in the corresponding figure or table legend), and the mean value of the corresponding parameter ± standard deviation is presented. In some cases, the level of significance of the differences when comparing results was evaluated by means of the Student’s t test with α = 0.05.

### Nucleotide Sequence Accession Numbers

The sequences of the GlucoBrick modules were deposited in the GenBank database with the GenBank accession numbers KU886714 (GlucoBrick Module I) and KU886715 (GlucoBrick Module II).

### ACKNOWLEDGMENTS

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