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## Mixing and matching methylotrophic enzymes to design a novel methanol utilization pathway in *E. coli*

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## ABSTRACT

23  
24 One-carbon (C1) compounds, such as methanol, have recently gained attention as  
25 alternative low-cost and non-food feedstocks for microbial bioprocesses.  
26 Considerable research efforts are thus currently focused on the generation of  
27 synthetic methylotrophs by transferring methanol assimilation pathways into  
28 established bacterial production hosts. In this study, we used an iterative combination  
29 of dry and wet approaches to design, implement and optimize this metabolic trait in  
30 the most common chassis, *E. coli*. Through *in silico* modeling, we designed a new  
31 route that “mixed and matched” two methylotrophic enzymes: a bacterial methanol  
32 dehydrogenase (Mdh) and a dihydroxyacetone synthase (Das) from yeast. To identify  
33 the best combination of enzymes to introduce into *E. coli*, we built a library of 266  
34 pathway variants containing different combinations of Mdh and Das homologues and  
35 screened it using high-throughput <sup>13</sup>C-labeling experiments. The highest level of  
36 incorporation of methanol into central metabolism intermediates (e.g. 22% into the  
37 PEP), was obtained using a variant composed of a Mdh from *A. gernerii* and a codon-  
38 optimized version of *P. angusta* Das. Finally, the activity of this new synthetic  
39 pathway was further improved by engineering strategic metabolic targets identified  
40 using omics and modelling approaches. The final synthetic strain had 1.5 to 5.9 times  
41 higher methanol assimilation in intracellular metabolites and proteinogenic amino  
42 acids than the starting strain did. Broadening the repertoire of methanol assimilation  
43 pathways is one step further toward synthetic methylotrophy in *E. coli*.

44 KEYWORDS: One-carbon metabolism, Methanol, *Escherichia coli*, Synthetic  
45 methylotrophy.

46

47

## 48 1. INTRODUCTION

49 In the quest to replace fossil fuel-based processes with more sustainable bio-based  
50 ones, low-cost and easy to use fermentation substrates are of great interest.  
51 Commonly used feedstocks such as hydrolyzed starch and molasses have the  
52 disadvantage of competing with food supply, and lignocellulosic biomass requires  
53 costly pre-treatment. A promising alternative feedstock is methanol, an abundant and  
54 pure raw material that can be utilized directly in bacterial fermentation processes.  
55 Furthermore, methanol's higher degree of reduction means that it is more electron  
56 rich than carbohydrates and these extra electrons can be expected to enhance  
57 product yields during fermentation (Whitaker et al., 2015). Methanol is currently one  
58 of the top five commodity chemicals with a global production capacity of about 110  
59 million metric tons per year and a price similar to that of glucose  
60 (<http://www.methanol.org/>). Although methanol is mainly produced from fossil  
61 resources, a notable advantage is that it can be produced by polygeneration, as a  
62 product of any renewable resource that can be converted into an intermediated  
63 synthesis gas (syngas). This includes biomass, agricultural and timber waste,  
64 municipal solid waste, landfill gas, industrial waste and a number of other feedstocks  
65 (<http://enerkem.com/fr/>; <http://www.methanol.org/>). Bio-methanol can also be  
66 produced from the thermo-, electro- or photo- catalytic reduction of the notorious  
67 greenhouse gas CO<sub>2</sub>. These approaches, which are still under development, can  
68 provide a way to recycle emitted CO<sub>2</sub> creating a carbon neutral cycle and, at the  
69 same time, store renewable or (excess) energy (Simakov, 2017). All these factors  
70 make methanol an attractive feedstock for biorefineries and the concept of a  
71 methanol economy has received considerable attention (Olah, 2013; Schrader et al.,  
72 2009).

73 Methylootrophy is the capacity of certain prokaryote and eukaryote microorganisms to  
74 use reduced one-carbon (C1) compounds such as methanol as their sole source of  
75 carbon and energy. This metabolism includes: (i) the oxidation of methanol to  
76 formaldehyde; (ii) the oxidation of formaldehyde to CO<sub>2</sub>, and (iii) the assimilation of  
77 one carbon compounds, either formaldehyde or CO<sub>2</sub> or a combination thereof (Heux  
78 S. et al., 2018). The industrial-scale use of natural methylootrophs has already been  
79 attempted. In the 1970s, a process was developed to produce single-cell protein  
80 (SCP) from methanol (Matelbs and Tannenbaum, 1968; Windass et al., 1980), but  
81 the technology fell out of favor in the following decades because of the low prices of  
82 alternative sources such as soybean protein. Currently, the use of natural  
83 methylootrophs in bioprocesses is only seen in the production by methylootrophic  
84 yeasts of recombinant proteins such as enzymes, antibodies, cytokines, plasma  
85 proteins, and hormones (Ahmad et al., 2014). The production of small molecules and  
86 metabolites (e.g. PHAs (polyhydroxyalkanoates) and amino acids) is still at the proof-  
87 of-concept stage (Schrader et al., 2009). The main limitations to the use of natural  
88 methylootrophs in biotechnologies are our currently weak understanding of their  
89 cellular metabolism and physiology, and the general lack of genetic tools to modify  
90 them (Chung et al., 2010; Schrader et al., 2009). In contrast, *Escherichia coli* is a  
91 robust biotechnological chassis with a wide range of products and an extensive  
92 genetic toolbox (Becker and Wittmann, 2015). Engineering a methanol assimilation  
93 pathway in this microorganism has thus become a popular research topic.

94 Methylootrophy is quite challenging to engineer because all biomass production and  
95 energy requirements must be satisfied by a reduced C1 precursor. In addition, cells  
96 must be able to tolerate formaldehyde, a central but toxic compound in methanol  
97 metabolism, whose accumulation due to an imbalance between oxidation and  
98 assimilation in the pathway can be fatal for cells. Because formaldehyde oxidation is  
99 efficient, the main bottleneck is C1 assimilation, which is achieved through a cyclic

100 process involving a C1-acceptor to enable the formation of C-C bonds. Several  
101 attempts have been made to engineer synthetic methylotrophy in *E. coli* using  
102 naturally occurring cyclic pathways (Wang et al., 2020). Most of these involve the  
103 expression of three heterologous enzymes: a NAD<sup>+</sup>-dependent methanol  
104 dehydrogenase (Mdh) for the oxidation of methanol to formaldehyde together with  
105 hexulose phosphate synthase (Hps) and phosphohexuloisomerase (Phi) from the  
106 ribulose monophosphate (RuMP) cycle for formaldehyde fixation. The *in vivo*  
107 operation of this pathway in *E. coli* has been confirmed by isotope-labeling  
108 experiments, which showed that methanol carbons were incorporated into cellular  
109 material (Muller et al., 2015). Similar results have also been reported in other model  
110 organisms such as *Corynebacterium glutamicum*, *Pseudomonas putida* and  
111 *Saccharomyces cerevisiae* (as reviewed recently by (Heux S. et al., 2018)).  
112 Improvements in methanol assimilation have been achieved using different strategies  
113 such as (i) optimizing the cultivation medium (Gonzalez et al., 2018), (ii) lowering the  
114 thermodynamic and kinetic constraints associated with NAD-dependent methanol  
115 oxidation (Roth et al., 2019; Wu et al., 2016), (iii) improving formaldehyde  
116 assimilation (Price et al., 2016; Woolston et al., 2018), (iv) increasing carbon fluxes  
117 through the autocatalytic cycle (Bennett et al., 2018), and (v) coupling the activity of  
118 the RuMP cycle to the growth of the host microorganism and then using adaptive  
119 laboratory evolution (Chen et al., 2018; He et al., 2018; Meyer et al., 2018). However,  
120 none of these synthetic strains are able to grow on methanol alone. The reasons for  
121 this and the obstacles to overcome include regenerating the C1-acceptor, protecting  
122 the cells against formaldehyde toxicity, channeling the substrate so that it can be  
123 integrated directly into the central metabolism, and lowering energetic constraints.

124 The approach outlined here to tackle the exciting challenge of synthetic  
125 methylotrophy is to develop a hybrid of naturally occurring cyclic methanol  
126 assimilation pathways. Using a “mix and match” approach, we created a new

127 synthetic pathway combining Mdh, a methylotrophic enzyme of bacterial origin, with  
128 dihydroxyacetone synthase (Das), a methylotrophic enzyme from yeast. The  
129 engineered strain was then optimized in an iterative process using omics  
130 (transcriptomics, metabolomics and fluxomics) and modelling approaches to identify  
131 bottlenecks. Overall, this approach allows non-natural pathways to be explored and  
132 tested while offering new perspectives on synthetic methylotrophy in *E. coli*.

133

## 134 **2. RESULTS**

### 135 ***2.1. Selecting the best design for a methanol assimilation pathway***

136 Natural methylotrophs have developed multiple pathways that allow them to grow on  
137 methanol as the sole source of carbon and energy (Chistoserdova, 2011). From this  
138 metabolic diversity, we can estimate that there are more than 500 unique methanol  
139 assimilation pathways from methanol to biomass (Heux S. et al., 2018). To identify  
140 the best pathway for *E. coli* to consume methanol, we used FindPath, a tool that  
141 freely recombines a repertoire of existing reactions to create metabolic pathways  
142 (Vieira et al., 2014). FindPath uses a substrate-associated reaction database and flux  
143 balance analysis (FBA) based on a genome scale model (GSM) of the host to (i) find  
144 all the possible pathways, and (ii) rank them according to their length and the  
145 predicted growth rate on the substrate of interest. The tool identified two equally  
146 efficient synthetic routes: the already well-studied RuMP-based pathway involving the  
147 bacterial enzymes Mdh, Hps and Phi, and a hybrid metabolic pathway, involving  
148 methylotrophic bacterial Mdh and methylotrophic yeast derived Das (Figure 1). The  
149 latter is a transketolase that catalyzes the fixation of formaldehyde on xylulose 5-  
150 phosphate (Xu5P) to form glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone  
151 (DHA) in the xylulose monophosphate (XuMP) cycle in methylotrophic yeasts. The  
152 GSM-predicted growth rate of *E. coli* on methanol with this pathway is 0.34 h<sup>-1</sup>. The

153 predicted fluxes show that this optimal growth rate is achieved when 16% of the  
154 methanol is incorporated into the biomass with the rest being used to recycle the C1  
155 acceptor, Xu5P. No flux through the methanol oxidation pathway (i.e. through FmA &  
156 B) was predicted. In addition, the GSM predicted that Xu5P would be recycled by  
157 fructose-6-phosphate aldolase and transaldolase (FSA/TAL pathway variant) rather  
158 than by fructose 1,6-bisphosphate aldolase with transaldolase (FBA/TAL variant), or  
159 by sedoheptulose biphosphatase (FBA/SBP variant) (Supplementary Figure S1). No  
160 ATP is required for Xu5P regeneration in the FSA/TAL variant, while the other two  
161 metabolic variants require two ATP molecules (Supplementary Figure S1). In  
162 comparison, in the synthetic RuMP based pathway, the C1 acceptor, ribulose-5-  
163 phosphate, is recycled using one or two ATP molecules. However, in term of  
164 assimilation no ATP is required in the synthetic RuMP based pathway while one  
165 equivalent of ATP is needed to phosphorylate DHA into DHAP (Supplementary  
166 Figure S1). Considering both recycling and assimilation, the synthetic RuMP pathway  
167 requires the same amount of ATP with the FBA/TA variant and one more ATP with  
168 the FBA/SBP variant compared to the FSA/TA variant which can occur only with the  
169 proposed pathway (Woolston et al., 2018). Finally, the transketolase activity of Das  
170 may contribute to the regeneration of the C1 acceptor, making our pathway partially  
171 independent of the pentose phosphate pathway (PPP). This has been demonstrated  
172 recently in methylotrophic yeasts (Russmayer et al., 2015).

173

## 174 **2.2. Screening for the best matching methylotrophic enzymes**

175 To identify the combination of enzymes that would optimize the *in vivo* activity of the  
176 pathway in *E. coli*, we built a combinatorial library of *mdh* and *das* homologues  
177 derived from native and non-native methylotrophs (bacteria and yeasts). Starting with  
178 the query protein sequences of *Bacillus methanolicus* PB1 Mdh2 and *Pichia angusta*



179 Das, a BLAST search with an identity cut-off at 50% was used with the software CD-  
180 HIT to filter out and cluster homologous templates (see Materials and Methods  
181 section). This threshold ensured that only functional homologues were identified  
182 (Sangar et al., 2007). Some of the 12 prokaryotic Mdh sequences and 17 eukaryotic  
183 Das sequences selected in this way belong to genera known to contain  
184 methyloprophs, such as *Bacillus*, *Burkholderia* (Chistoserdova et al., 2009),  
185 *Acinetobacter* (Del Rocío Bustillos-Cristales et al., 2017), *Pichia* and *Candida*  
186 (Supplementary Figure S2). We added an Mdh from *Bacillus stearothersophilus* and  
187 a *P. angusta* methanol oxidase (MOX), since both have been reported to have good  
188 affinity for methanol (Kms of 20 mM and 0.4 mM, respectively) (Shleev et al., 2006;  
189 Whitaker et al., 2017). Finally we added a Das from *Mycobacterium* with a high  
190 affinity for formaldehyde (Km of 1.86 mM) (Ro et al., 1997) and an *E. coli* codon-  
191 optimized version of *P. angusta* Das. The Mdh and Das genes were respectively  
192 cloned into the low-copy plasmid pSEVA424 and the middle-copy plasmid pSEVA134  
193 (Silva-Rocha et al., 2013). All the selected sequences were assembled in a library of  
194 266 combinations of genes (14 Mdh sequences \* 19 Das sequences) and  
195 transformed using a robotic platform (Supplementary Figure S3). To prevent the  
196 induction of the formaldehyde detoxification pathway (formaldehyde to CO<sub>2</sub>) (Figure  
197 1), we used an *E. coli* strain  $\Delta frmA$ , deleted for the formaldehyde dehydrogenase i.e.  
198 the first gene of this pathway. Enzyme expression were measured at two reasonable  
199 *E. coli* growth temperatures i.e. 30°C and 37°C. The higher expression obtained at  
200 30°C overnight (Supplementary Figure S4) led us to use this temperature for all  
201 subsequent experiments.

202 To analyze the performance of the 266 different enzyme combinations, methanol  
203 incorporation was measured for each pathway variant using dynamic <sup>13</sup>C-labeling  
204 experiments as shown in Supplementary Figure S3. We used the <sup>13</sup>C-labeling  
205 incorporation into the phosphoenolpyruvate (PEP) as a proxy for methanol

206 assimilation since PEP is one of the first multi-carbon products of methanol  
207 assimilation (Figure 1). The  $^{13}\text{C}$ -enrichment of PEP measured for each combination  
208 of Mdh and Das is shown in Figure 2A and Supplementary Table S2.  $^{13}\text{C}$   
209 enrichments of between 1% to 5% were observed for the combinations involving the  
210 *das* genes of *Pichia pastoris*, *Verruconis gallopava*, *Scedosporium apiospermum*,  
211 *Rasamsonia emersonii*, *Fonsecaea erecta* and *Kuraishia capsulata*. In comparison,  
212 combinations involving the *das* from *P. angusta*, had  $^{13}\text{C}$  enrichments two to twelve  
213 times higher, up to 22% for the codon-optimized version, *P. angusta* (opt),  
214 representing an average 2.4-fold increase in  $^{13}\text{C}$ -isotopic enrichment in PEP  
215 compared with the wild type (Supplementary Table S2). These results are consistent  
216 with the more stable expression of the codon-optimized version of Das, compared  
217 with the wild type version (Supplementary Figure S5).

218 We then investigated whether methanol assimilation could be increased by  
219 optimizing the expression of the Das enzymes which led to low- or non-labeled PEP.  
220 The codon-optimized Das genes from *Candida boidinii*, *P. methanolica* A, *Aspergillus*  
221 *fumigatus* Z5, *D. hansenii*, *R. emersonii* and *K. capsulata* were synthesized and  
222 individually co-expressed with *A. gernerii* Mdh. No significant improvement in  $^{13}\text{C}$ -  
223 enrichment was observed compared with the native sequences, except for codon-  
224 optimized *R. emersonii* Das, whose PEP labeling was twice as high (4% vs 2%  $^{13}\text{C}$ -  
225 enrichment) (Supplementary Table S3).

226 Labeling was observed for all these combinations regardless of the nature of the  
227 Mdh, suggesting Das compensated for the generally poor kinetic properties of the  
228  $\text{NAD}^+$ -dependent Mdh enzymes (Brautaset et al., 2013; Krog et al., 2013) by shifting  
229 the equilibrium toward methanol oxidation and subsequent formaldehyde  
230 assimilation. However, the level of  $^{13}\text{C}$ -incorporation was not linked with the  
231 expression levels of either Mdh or Das (Figure 2A and Supplementary Figure S5). It

232 is also worth noting that although *B. stearothermophilus* Mdh and *Mycobacterium*  
233 Das have favorable *in vitro* and *in vivo* activities (Ro et al., 1997; Whitaker et al.,  
234 2017), and were well-expressed in *E. coli* (Supplementary Figure S5), they led to  
235 very low <sup>13</sup>C-enrichment (< 3%) in most of the tested combinations (Figure 2A).

236 Finally, the highest methanol incorporation was achieved when either *A. gernerii* Mdh  
237 or *Burkholderia sp. TSV86* Mdh was expressed in combination with *P. angusta* Das  
238 (opt). With these combinations, the <sup>13</sup>C-enrichments of PEP were respectively 13 and  
239 12 times higher than with the query pathway (*B. methanolicus* PB1 Mdh2 / *P.*  
240 *angusta* Das). In particular, the fractions of PEP with one <sup>13</sup>C atom were 31% and  
241 32% and reached 17% and 13.7% for two <sup>13</sup>C atoms, respectively (Figure 2B). The  
242 incorporation of more than one labeled carbon into PEP demonstrates that the  
243 recycling of Xu5P is functional in both combinations.

244 The higher <sup>13</sup>C-enrichment obtained for the combination involving *A. gernerii* Mdh and  
245 *P. angusta* Das (opt) led us to use these enzymes for subsequent experiments.  
246 Although this is the best matching of enzymes, the fact that 100% PEP labeling was  
247 not achieved indicates that methanol alone cannot supply all the carbon atoms  
248 required for molecular assembly and that pure methylotrophic growth is not yet  
249 possible with this pathway.

### 250 **2.3. Characterizing the cellular behavior of the synthetic methylotroph**

251 In order to uncover the specific make-up of the new synthetic methylotrophic *E. coli*  
252 strain with regard to methanol utilization, we performed a physiological and  
253 transcriptomic analysis of the strain expressing *A. gernerii* Mdh and *P. angusta* Das  
254 (opt) grown on xylose with and without additional methanol (Table 1, Figure 3). For  
255 this analysis, both genes were cloned into the pSEVA424 vector as a single operon  
256 to avoid the metabolic burdening of the cells with double-antibiotic selection (Silva et  
257 al., 2012). Similar levels of <sup>13</sup>C-methanol incorporation were measured in the single

258 and double plasmid strains after 90 min culture with methanol, but labeling continued  
259 to increase in the double plasmid strain up to twice the level observed in the single  
260 plasmid strain (Supplementary Figure S6). This can be explained by a decrease in  
261 Das levels when the gene is expressed from the low-copy plasmid pSEVA424 (10–15  
262 copies/cell) instead of the middle-copy pSEVA131 plasmid (20–30 copies/cell) (Silva-  
263 Rocha et al., 2013).

264 The physiological response to methanol of the synthetic strain expressing the  
265 assimilation pathway on one plasmid is given in Table 1. Both the growth rate (+54%)  
266 and the specific xylose consumption rate (+45%) were higher in the methanol-  
267 supplemented medium than when cells were grown on xylose alone. Formate was  
268 only observed in the presence of methanol (Table 1) and its production increased  
269 once xylose was depleted (Figure 3A). In contrast, methanol consumption could not  
270 be formally assessed since the decrease in concentration occurred at a similar rate  
271 as evaporation and fell within the error range of the NMR instrument (4% of the  
272 measured value). These results clearly indicate a positive effect of methanol on the  
273 rate of xylose uptake, and thus on growth, but also show that formaldehyde was  
274 oxidized into formate even though the first step of this pathway had been deleted (i.e.  
275 *frmA*).

276 To characterize the cellular response of the synthetic strain to methanol, a  
277 transcriptional analysis was performed during exponential growth on xylose, with or  
278 without methanol supplementation. Specifically, we looked at the expression of the  
279 genes involved in methanol metabolism (Figure 3B). In the presence of methanol the  
280 *frmR* and *frmB* genes were strongly up-regulated. Because FrmR is a formaldehyde  
281 sensing factor which regulates expression of the *frmRAB* operon, *frmR* and *frmB* up-  
282 regulation indicates the production of formaldehyde into the cells which was expected  
283 from the presence of methanol (Figure 3B). However, the production of formate in the

284  $\Delta frmA$  strain (Table 1) suggests the presence of a promiscuous alcohol  
285 dehydrogenase that replaces FrmA in the reaction from formaldehyde to S-  
286 formylglutathione, which is then converted to formate by FrmB. Up-regulation of *dhaL*  
287 and *dhaM*, which encode the dihydroxyacetone kinase (DAK) pathway, was also  
288 observed in presence of methanol, (Figure 3B). Because the expression of the  
289 *dhaKLM* operon is induced by DHA (Bächler et al., 2005), this confirms the presence  
290 of DHA in the cells and thus the co-assimilation of methanol with xylose. However,  
291 the genes encoding alternative DHA assimilation routes (i.e. the glycerol (*gldA*, *glpK*  
292 and *glpD*) and the FSA (*fsaA* and *fsaB*) pathways) were not transcriptionally activated  
293 or even down-regulated (Figure 3B). These results are consistent with the conclusion  
294 of Peiro et al. that DHA is mainly assimilated via the dihydroxyacetone kinase (DAK)  
295 (Peiro et al., 2019). However, they appear to contradict those of the flux balance  
296 analysis that predict that Fsa may be involved in the regeneration of the C1 acceptor.  
297 Finally, the gene encoding the transketolase *tktA* was up-regulated on methanol  
298 (Figure 3B). This enzyme catalyzes the formation of Xu5P, which plays a key role in  
299 the cyclic operation of our synthetic pathway. However, Xu5P is also the entry point  
300 of xylose in the metabolism and, interestingly, the presence of methanol improved the  
301 expression of *xylE* involved in its transport through the cellular membrane (Figure  
302 3B), particularly during the exponential growth phase. This result corroborates the  
303 higher specific xylose uptake rate observed when the synthetic *E. coli* strain was  
304 grown in media supplemented with methanol (Table 1).

305 Overall, these data demonstrate that methanol can be assimilated by the new  
306 synthetic *E. coli* strain and identified genetic engineering targets to limit its  
307 dissimilation and improve the cyclic operation of the pathway.

308

309 **2.4. Optimizing the methylotrophic chassis**

310 The choice of the genes (Figure 4B) to be targeted in the current strain (Strain 1) to  
311 engineer a superior methanol assimilation phenotype was based on: (i)  
312 computational prediction highlighting the key role of Fsa for the regeneration of the  
313 C1 acceptor, XU5P, making the DHA a key junction between assimilation and  
314 recycling in our synthetic strain; (ii) a previous study which demonstrated that  
315 increasing expression of *fsaB* and *gldA* leads to an improved DHA assimilation (Peiro  
316 et al., 2019); (iii) the above transcriptomic analysis identifying the genes encoding  
317 enzymes for formaldehyde dissimilation, xylulose-5-phosphate recycling and  
318 alternative DHA assimilation pathways as potential targets to boost the assimilation  
319 of methanol in our synthetic strain. Strain 2 was built by knocking out the entire  
320 *frmRAB* operon to avoid drainage of formaldehyde to the detoxification pathway.  
321 Strain 3 was built by knocking out the *frmRAB* operon in a  $\Delta ptsA::kan$  mutant. In this  
322 strain the *ptsA* gene is replaced by a kanamycin cassette leaving the downstream  
323 genes within the same operon, *gldA* and *fsaB*, under the control of the kanamycin  
324 promoter. As previously observed in a  $\Delta ptsA::kan$  strain (Peiro et al., 2019), a full  
325 activation of both the GLD and FSA pathways is expected in the strain 3. In strain 4  
326 finally, *tktA*, a gene encoding a key enzyme in the regeneration of Xu5P, was  
327 overexpressed to promote this process.

328 To study the impact of these genetic modifications on methanol assimilation, the  
329 genealogy of the new rationally designed strains was characterized by following the  
330 incorporation of  $^{13}\text{C}$ -methanol atoms into intracellular and extracellular metabolites  
331 (Figure 4 and controls in supplementary Figure S7). Knocking out the *frmRAB* operon  
332 (strain 2) resulted in a small increase in  $^{13}\text{C}$ -methanol incorporation in all the  
333 measured intracellular metabolites compared with the starting  $\Delta frmA$  strain 1 (Figure  
334 4A), in line with measurements of the extracellular production of formate (Figure 4C).  
335 Upon xylose depletion in the medium,  $^{13}\text{C}$ -formate production was detected in strain  
336 1 and increased constantly during the stationary phase. In contrast, strain 2 did not

337 produce  $^{13}\text{C}$ -formate, even after several hours in the stationary phase. In this strain,  
338 we observed a small but significant increase of the  $^{13}\text{C}$ -enrichment of the pool of 2  
339 and 3 phosphoglycerate (23PG, + 2%) and phosphoenolpyruvate (PEP, + 1.9%) and,  
340 more specifically, of the fractions with one  $^{13}\text{C}$  atom (M1) compared with the starting  
341 strain (strain 1) (Figure 4A). The most significant improvement in methanol  
342 assimilation was observed in strain 3, in which *fsaB* and *gldA* were overexpressed. In  
343 line with the activation of the glycerol pathways in strain 3, a large fraction of glycerol-  
344 3-phosphate (GLYC3P) with two  $^{13}\text{C}$  atoms (M2) was measured resulting in an  
345 increase of 11.5 % of the  $^{13}\text{C}$ -enrichment compared with strain 1. GLYC3P is an  
346 important precursor of membrane constituents and therefore of biomass. In strain 3,  
347 all the measured central metabolites had more than one  $^{13}\text{C}$  atom (Figure 4A), which  
348 can only have resulted from recycling of the C1 acceptor, XU5P. This is in  
349 accordance with the computational prediction that Fsa plays a key role in the cyclic  
350 operation of the synthetic pathway (Figure 1). Strain 4, in which *tktA* was  
351 overexpressed, showed higher  $^{13}\text{C}$ -methanol incorporation into F6P. The fraction  
352 carrying two  $^{13}\text{C}$  atoms was twice as high in F6P compared with strain 3 and traces  
353 of F6P containing three  $^{13}\text{C}$  atoms (M3) were also detected. In addition, traces of  
354 labeling were measured in the pentose phosphate pool (P5P) containing  
355 XU5P. However,  $^{13}\text{C}$ -formate was once again detected in this strain (Figure 4C). This  
356 might derive from another reaction catalysed by the 3,4-dihydroxy-2-butanone-4-  
357 phosphate synthase, RibB, which converts ribulose-5-phosphate (Ru5P) into  
358 formate (Richter et al., 1992). By overexpressing *tktA*, the pool of Ru5P is indeed  
359 expected to be higher.

360 To confirm that carbon molecules originating from methanol were used in  
361 biosynthetic pathways, we also analyzed  $^{13}\text{C}$  incorporation into proteinogenic amino  
362 acids after 48h of cultivation on  $^{13}\text{C}$ -methanol (Supplementary Figure S8). Low but  
363 significant levels of  $^{13}\text{C}$  were found. In agreement with the labeling observed in the

364 glycolytic and TCA intermediates, labeling was also observed in their derived amino  
365 acids i.e. serine (SER, derived from glyceraldehyde-3-phosphate), alanine (ALA,  
366 derived from pyruvate), aspartate and glutamate/glutamine (ASP and GLX, derived  
367 respectively from oxaloacetate and  $\alpha$ -ketoglutarate). As expected from the small  
368 amounts of labeled carbon in the P5P pool, no labeling was found in histidine (HIS),  
369 which is derived from ribose-5-P. However, some labeling was detected in  
370 phenylalanine (PHE), which is derived from erythrose-4-phosphate. The fraction of  
371 labeled carbons increased systematically from strain 1 to strain 4 and, more  
372 importantly, a fraction of proteinogenic amino acids were found to carry more than  
373 one  $^{13}\text{C}$  atom (Supplementary Figure S8).

374

### 375 **3. DISCUSSION**

376 Methanol is an attractive feedstock for the production of fuels and chemicals but  
377 engineering a C1 fixation pathway into an industrially relevant microorganism, such  
378 as *E. coli*, remains challenging. To tackle this problem, this article describes a new  
379 computationally designed pathway as an alternative to the well-studied RuMP based  
380 pathway (Bennett et al., 2018; Chen et al., 2018; Gonzalez et al., 2018; He et al.,  
381 2018; Meyer et al., 2018; Muller et al., 2015; Price et al., 2016; Whitaker et al., 2015;  
382 Woolston et al., 2018). This new pathway is a hybrid of naturally occurring cyclic  
383 methanol assimilation pathways and consists of a Mdh from *A. gernerii* in combination  
384 with a codon-optimized version of *P. angusta* Das. Although the new pathway does  
385 not allow the cell to grow on methanol alone, 22% incorporation of methanol carbon  
386 was observed in the multi-carbon compound PEP. This is similar to the values  
387 measured previously in a synthetic methylotrophic *E. coli* strain expressing cyclic  
388 RuMP based-pathways and cultivated under comparable conditions i.e. without yeast  
389 extract (Supplementary Table S4). Importantly, this article reports the discovery of



390 two novel NAD-dependent alcohol dehydrogenases from Gram-negative, mesophilic,  
391 non-methylotrophic organisms (*A. gernerii* and *Burkholderia* sp.) with significant *in*  
392 *vivo* affinity for methanol. Representatives of the Burkholderia order have recently  
393 been recognized as true facultative methylotrophs (Chistoserdova et al., 2009) and  
394 one NAD-dependent Mdh from this order has the highest *in vitro* affinity for methanol  
395 reported to date (Woolston et al., 2018; Wu et al., 2016; Yu and Liao, 2018). In our  
396 setting, the two novel Mdhs performed better *in vivo* than the Mdhs from *B.*  
397 *methanolicus* and *B. stearothersophilus* which were used previously to implement  
398 methylotrophy in *E. coli* (Bennett et al., 2018; Chen et al., 2018; Gonzalez et al.,  
399 2018; Kim et al., 2020; Meyer et al., 2018; Muller et al., 2015; Whitaker et al., 2017).  
400 However, the efficiency of the pathway was mostly improved by using a codon-  
401 optimized version of Das, indicating that this enzyme is very likely rate-limiting for  
402 methanol assimilation. Since Das was not overexpressed as much as Mdh was  
403 (Supplementary Figure S4), further increasing its expression should also increase  
404 methanol assimilation.

405 Our iterative process of strain analysis and engineering combining omics and  
406 modelling approaches was decisive in the selection of strategic genetic targets to  
407 maximize methanol assimilation. The final optimized strain incorporated 1.5 to 5.9  
408 times more methanol — as measured by <sup>13</sup>C-enrichment and depending on the  
409 metabolite — than did the starting strain. A maximum <sup>13</sup>C-enrichment of 37% was  
410 achieved in GLYC3P. In addition, the increase in the number of labeled carbons per  
411 molecule for most metabolites shows that cyclic operation of the synthetic pathway  
412 was improved in the final strain. Finally, the presence of labeling in biomass  
413 constituents showed that carbon molecules originating from methanol were not only  
414 assimilated into the central metabolism but also used in biosynthetic pathways. This  
415 is evidence of true methanol metabolism and confirms the establishment of  
416 methylotrophy in this *E. coli* strain. In the optimized strain, the most significant

417 improvement was achieved by activating alternative DHA assimilatory pathways. This  
418 is consistent with a previous study demonstrating that the specific DHA uptake rate in  
419 a similar engineered strain was increased by 60% (Peiro et al., 2019). We further  
420 improved methanol assimilation in the synthetic strain by overexpressing a  
421 transketolase and, therefore, improving the recycling of the C1 acceptor. This is in  
422 agreement with the conclusion of a previous study that expressing the non-oxidative  
423 pentose phosphate pathway (PPP) from *B. methanolicus* improves methanol  
424 assimilation in a synthetic *E. coli* methylotroph (Bennett et al., 2018).

425 Finally, we also observed that methanol improved the growth of our synthetic strain  
426 on xylose by up-regulating the genes involved in xylose transport through the cellular  
427 membrane. Up-regulation of genes encoding transmembrane transporters in the  
428 presence of methanol has also been observed in *S. cerevisiae* (Espinosa et al.,  
429 2019). The chemical properties of methanol are known to modify the physical  
430 properties of cell membranes, such as their fluidity (Joo et al., 2012). These changes  
431 can be perceived by the cells and trigger the expression of genes that are involved in  
432 the acclimation of cells to new conditions (Los and Murata, 2004).

433 In this work, we successfully created an *E. coli* strain able to efficiently assimilate  
434 methanol through a brand new synthetic metabolic pathway. However, there is still  
435 room for optimization and our results suggest that the overall metabolic capacity for  
436 methanol can be improved in several ways. For example, one could (i) improve the  
437 expression of Das, (ii) block all the dissimilatory pathways, (iii) improve the recycling  
438 of the C1 acceptor, and (iv) coordinate the catabolic pathway with the overall cellular  
439 infrastructure by engineering methanol-sensitive elements to improve the global  
440 response to the substrate (Rohlhill et al., 2017) or by directed evolution (Chen et al.,  
441 2018; He et al., 2018; Meyer et al., 2018). However, a recent study demonstrating the  
442 slow growth (doubling time of 54 h) on a mixture of methanol and CO<sub>2</sub> of an *E. coli*

443 strain expressing a linear methanol assimilation pathway (Kim et al., 2020) raises  
444 questions about the relevance of establishing methylotrophy in *E. coli* using cyclic  
445 pathways. Arguments in favor of pursuing the quest for growth on pure methanol  
446 using cyclic pathways are (i) the independence of such pathways from other carbon  
447 sources, and (ii) a recent study reporting an *E. coli* strain expressing an autotrophic  
448 cycle capable of producing all its biomass carbon from CO<sub>2</sub> (Gleizer et al., 2019).

449

## 450 **4. Materials and Methods**

### 451 **4.1 Reagents**

452 All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless noted  
453 otherwise. Unlabeled methanol ( $\geq 99.9\%$ , LC-MS grade) was purchased from  
454 Honeywell (Muskegon, MI, USA). Isotopically labeled <sup>13</sup>C-methanol (99% <sup>13</sup>C) was  
455 purchased from Eurisotop (Saint-Aubin, France). Phusion® DNA polymerase and  
456 restriction enzymes were purchased from New England Biolabs Inc. (Beverly, MA,  
457 USA).

### 458 **4.2 Bacterial strains and culture media**

459 All the strains, plasmids, primers and synthetic gene constructs used in this study are  
460 listed in Supplementary Table S5. *E. coli* DH5 $\alpha$  was used for plasmid construction  
461 and propagation whereas *E. coli* BW25113 was used for methanol assimilation. *E.*  
462 *coli* BW25113 $\Delta$ frma::*neo* was obtained from the Keio collection and the Flp  
463 recognition target (FRT)-flanked kanamycin cassette was removed using Flp  
464 recombinase from pCP20 plasmid (Cherepanov and Wackernagel, 1995). After  
465 recombination, loss of pCP20 was confirmed by re-streaking on ampicillin, and  
466 removal of the resistance cassette was confirmed by polymerase chain reaction  
467 (PCR). For operon construction, *A. gernerii* Mdh and *P. angusta* Das genes,

468 containing RBS and a 6xHis tag, were amplified from the pSEVA plasmids using  
469 primers P91&P92 and P83&P93, respectively. The two fragments, designed to  
470 overlap by 35 bp, were joined by overlapping PCR. The complete Mdh-Das operon  
471 was subsequently cloned into the pSEVA424 vector using primers P102&P103 and  
472 the In-Fusion® HD kit (Takara Bio, Otsu, Japan). The  $\lambda$  red recombination method  
473 (Datsenko and Wanner, 2000) was used to generate knockout strains  $\Delta$ ptsA (primers  
474 P129&P130) and  $\Delta$ frmRAB (primers P145&P146). The introduced antibiotic  
475 resistance cassettes were removed using the FRT/FLP recombination system  
476 (Cherepanov and Wackernagel, 1995). All constructs were subsequently verified by  
477 colony PCR and sequencing (GATC, Konstanz, Germany).

478 All *E. coli* strains harboring plasmids were propagated in Luria-Bertani (LB) medium  
479 or M9 minimal medium containing the appropriate antibiotics. The composition of the  
480 M9 minimal medium was as follows (in  $\text{g}\cdot\text{L}^{-1}$ ): 18  $\text{Na}_2\text{HPO}_4$ , 3.13  $\text{KH}_2\text{PO}_4$ , 0.53  $\text{NaCl}$ ,  
481 2.11  $\text{NH}_4\text{Cl}$ , 0.49  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ , 0.00438  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ , 0.1 thiamine hydrochloride,  
482 trace elements ( $\text{mg L}^{-1}$ ) 15  $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ , 4.5  $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ , 0.3  $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$ , 1  
483  $\text{MnCl}_2$ , 1  $\text{H}_3\text{BO}_3$ , 0.4  $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$ , 3  $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ , 0.3  $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$ . The  
484 antibiotics were added when necessary in the following concentrations: ampicillin  
485 (Amp, 100  $\mu\text{g}/\text{ml}$ ), kanamycin (Kan, 50  $\mu\text{g}/\text{ml}$ ), streptomycin (Strp, 50  $\mu\text{g}/\text{ml}$ ). The  
486 optical density at 600 nm (OD600) was measured using a GENESYS 6™  
487 spectrophotometer (Thermo Scientific).

#### 488 **4.3 In silico design of the synthetic pathway**

489 The synthetic pathway for methanol assimilation was designed using the software  
490 FindPath (Vieira et al., 2014). The workflow starts with the creation of a substrate-  
491 associated reaction database based on the literature and available metabolic  
492 databases. This database consists of reactions involving the target molecule (in our  
493 case, methanol). The database is then converted into a model that is subsequently

494 used to compute elementary flux modes (EFMs), i.e., all the possible flux distributions  
495 in a metabolic network under steady state conditions. Among these EFMs, the best  
496 pathways are selected and ranked according to their efficiency. Finally, the best  
497 module combinations for efficient methanol conversion were identified. In our case,  
498 the methanol database encompassed more than 100 reactions steps and 100  
499 metabolic compounds involved in methanol metabolism. For each reaction, the  
500 genes, reaction, EC number, KEGG name, localization, and reversibility were  
501 reported. Finally, the model was built by bringing together all the reactions along with  
502 the transporters and cofactor recycling, i.e. 47 reactions and 114 metabolites. Using  
503 this model, 10000 EFMs were generated of which 85 allowed the conversion of  
504 methanol into *E. coli* metabolites. From these, 20 efficient EFMs were selected, i.e.  
505 those involving a small number of reactions and with low cofactor consumption (ATP,  
506 NAD(P)H). The hypothesis was that their introduction into the host would require little  
507 genetic effort (the number of genes being correlated with the number of reactions)  
508 and would have little or no effect on the host's energy and redox machinery. The  
509 reactions composing the 20 EFMs were implemented in a genome scale model *E.*  
510 *coli* (iAF1620). Finally, the biomass yields on methanol of each of the 20 EFMs were  
511 simulated using *in-silico* flux balance analysis (FBA),.

#### 512 **4.4 Library generation by combinatorial assembly**

513 A BLAST search against UniRef50 (Suzek et al., 2014) using *B. methanolicus* PB1  
514 Mdh2 (UniProt ID: I3DVX6) and *P. angusta* Das (UniProt ID: P06834) as query  
515 sequences returned two clusters with 177 and 230 members, respectively. The  
516 sequence clustering tool H-CD-HIT (Huang et al., 2010) was used to hierarchically  
517 merge similar sequences at varying levels of sequence identity. Proteins were first  
518 clustered at a high identity (90%) before the non-redundant sequences were further  
519 clustered at a low identity (80% and eventually 70%). Among the representatives of

520 the different clusters, we selected 12 putative Mdh variants and 17 putative Das  
521 variants from aerobic and mesophilic microorganisms. The corresponding Mdh  
522 genes, as well as the *Bacillus stearothermophilus* Mdh (Dowds et al., 1988) and  
523 *Pichia angusta* Mox genes (Shleev et al., 2006), were cloned in the expression vector  
524 pSEVA424 (Silva-Rocha et al., 2013) between restriction sites AvrII and NotI. The  
525 selected Das genes, plus the *Mycobacterium* Das gene (Ro et al., 1997) and an *E.*  
526 *coli* codon-optimized version of P06834, were cloned in the expression vector  
527 pSEVA134 (Silva-Rocha et al., 2013) between restriction sites AvrII and SpeI. All the  
528 constructs were synthesized and cloned by BaseClear (Leiden, The Netherlands).  
529 The same ribosome binding site (RBS) (AGGAGGAAAAACAT) and 6xHis tag was  
530 used for all the genes. The two gene libraries were co-transformed in the  
531 BW25113ΔfrmA::frr strain using the rubidium chloride method (Green and Rogers,  
532 2013) and plated on LB-Amp-Strp plates (Supplementary Figure S3).

#### 533 **4.5. Dynamic <sup>13</sup>C-labeling incorporation**

534 To study the incorporation of <sup>13</sup>C-methanol into intracellular metabolites and  
535 proteinogenic amino acids, cells were first cultured in M9 minimal medium in the  
536 presence of 15 mM xylose, antibiotics and 0.1 mM IPTG, in 96-deep-well plates, at  
537 30°C and 220 rpm until exponential phase (OD<sub>600</sub> = 0.5-1). The cells were then  
538 centrifuged at 4400g for 3 min and resuspended in M9 minimal medium with reduced  
539 (five times less) phosphate and sulfate, IPTG, antibiotics, and <sup>13</sup>C-methanol (655  
540 mM). The methanol concentration was chosen to be sufficiently above the K<sub>m</sub> of  
541 Mdh.

542 After 90 and 180 min incubation at 30°C and 220 rpm, intracellular metabolites were  
543 sampled as follows: 120 μL of culture was taken and mixed with 1 mL of cold (-20°C)  
544 acetonitrile:methanol:water:formic acid (40:40:20:0.1) extraction solution. The  
545 samples were vacuum-dried overnight. The next morning, dried metabolites were

546 resuspended in 120  $\mu$ L water, centrifuged at 16,000  $\times$  g for 2 min, and injected into  
547 the LC-MS. Central metabolites were separated on a Dionex™ IonPac AS11-HC  
548 anion-exchange column (250  $\times$  2 mm) equipped with an AG11 guard column (50  $\times$  2  
549 mm) with KOH as the mobile phase using a Dionex™ ICS-5000+ Reagent-Free™  
550 HPIC™ system (Thermo Fisher Scientific™, Sunnyvale, CA, USA). Separation of  
551 PEP shown in Figure 2 was carried out with a flow rate set at 0.38 ml/min and the  
552 following elution gradient: 0 min, 0.5 mM; 1 min, 0.5 mM; 9.5 min, 4.1 mM; 14.6 min,  
553 4.1 mM; 24 min, 9.65 mM; 31.1 min, 100 mM and 43 min, 100 mM. For separation of  
554 central metabolites shown in Figure 4, the elution gradient was as follows: 0 min, 7  
555 mM; 1 min, 7 mM; 9.5 min, 15 mM; 20 min, 15 mM; 30 min, 45 mM; 33 min, 70 mM;  
556 33.1 min, 100 mM; 42 min, 100mM; 42.5 min, 7 mM and 50 min, 7 mM. Metabolites  
557 were detected using a Thermo Scientific™ LTQ Orbitrap Velos™ mass spectrometer  
558 in negative electrospray ionization mode. The spray voltage was 2.7 kV, the capillary  
559 and desolvation temperatures were 350°C, and the maximum injection time was 50  
560 msec. The spectrometer was operated in full-scan mode at a resolution of 60,000  
561 (400 m/z).

562 After 48 h of incubation at 30°C and 220 rpm, proteinogenic amino acids were  
563 sampled as follows: the plates were centrifuged at 4400g for 3 min and the  
564 supernatant was removed. To release protein-bound amino acids from cellular  
565 proteins, the cell pellets collected were hydrolyzed for 15 h with 6N HCl at 100°C.  
566 HCl was evaporated at low pressure (20 mbar, room temperature). Biomass  
567 hydrolysates were washed twice in water using the same evaporation method. The  
568 dried hydrolysates were resuspended in 200  $\mu$ L water and centrifuged. A 10-fold  
569 dilution was prepared, and samples were analyzed by LC-HRMS. Proteinogenic  
570 amino acids were separated on a Supelco™ HS F5 DISCOVERY column (150  $\times$  2.1  
571 mm; 5  $\mu$ m) equipped with a SUPELGUARD KIT HS F5 guard column (20  $\times$  2.1 mm;  
572 5  $\mu$ m) with 0.1% formic acid (solvent A) and 0.1% acetonitrile/formic acid (solvent B)

573 as the mobile phase using a UHPLC Vanquish system (Thermo Fisher Scientific™,  
574 Sunnyvale, CA, USA). The flow rate was set to 0.25 ml/min and the elution gradient  
575 was (% B): 0 min at 2%, 2 min at 2%, 10 min at 5%, 15 min at 35%, 20 min at 100%,  
576 24 min at 100%, 24,1 min at 2% and 30 min at 100%. Metabolites were detected  
577 using a Thermo Scientific™ Orbitrap Q-Exactive+™ mass spectrometer in positive  
578 electrospray ionization mode, with a spray voltage of 5 kV, and capillary and  
579 desolvation temperatures of 250°C. The spectrometer was operated in full-scan  
580 mode at a resolution of 60,000 (400 m/z).

581 <sup>13</sup>C-carbon isotopologue distributions were identified by matching masses from the  
582 mass spectra (mass tolerance of 5 ppm) and retention times using the software  
583 TraceFinder (v. 4.1). The peaks of different isotopologues were integrated and  
584 corrected for the natural abundance and isotopic purity of the tracer using the  
585 software IsoCor (Millard et al., 2019). Levels of <sup>13</sup>C-isotopic enrichment were then  
586 determined as follows: <sup>13</sup>C-enrichement (%) =  $\sum(M_i \cdot i) / n$ , where n is the number of  
587 carbon atoms for the measured fragment and M<sub>i</sub> is the corrected abundance of the  
588 mass isotopologue.

#### 589 **4.6 Supernatant analysis**

590 Metabolite utilization and the production of the synthetic methylotroph were analyzed  
591 by quantitative 1D <sup>1</sup>H-NMR at 280 K using a zgpr30 sequence with water pre-  
592 saturation prior to acquisition on an Avance III 500 MHz spectrometer (Bruker,  
593 Rheinstetten, Germany) equipped with a 5 mm QPCI cryogenic probe head. The  
594 parameters were as follows: 286°K, 128K points, 8 s relaxation time, 2 dummy  
595 scans, 32 scans. Free induction decays (FIDs) were converted into frequency domain  
596 spectra by Fourier transform. All spectra were processed using the software TopSpin  
597 (v. 3.5). Phases were adjusted manually, baselines were adjusted automatically, and  
598 the spectra were aligned and quantified using 3-trimethylsilylpropionic-2,2,3,3-d<sub>4</sub> acid



599 sodium salt (TSP-d4, 1 mM) as a chemical shift and concentration standard. The  
600 concentrations of the different metabolites (xylose, methanol, formate, and acetate)  
601 were calculated with the following equation: concentration = integrated peak  
602 area\*TSP concentration\*dilution of the sample/number of protons in the molecule.  
603 For xylose, only the peaks corresponding to the anomeric protons were integrated.

#### 604 ***4.7 Transcriptomic analysis***

605 Cells were grown in flasks of M9 minimal media containing 15 mM xylose with or  
606 without 150 mM MeOH. At T1 (OD600 = 1, exponential phase) and T2 (OD600 = 2,  
607 stationary phase), 4 mL of each culture was centrifuged for 90 s at 14000 rpm before  
608 discarding the supernatant and immediately freezing the pellets in liquid nitrogen.  
609 Total RNA was extracted according to the Qiagen RNAeasy MiniKit procedure and  
610 quantified using a Nanodrop® spectrophotometer. Double-stranded complementary  
611 DNA (cDNA) synthesis and array processing were performed using the Agilent  
612 Technologies One-Color Microarray-Based Gene Expression Analysis protocol. The  
613 images were analyzed with the software DEVA (v. 1.2.1). All array procedures were  
614 performed using the GeT-Biopuces platform (<http://get.genotoul.fr/>). For each data  
615 set, corresponding to time point T1 or T2, the log<sub>2</sub> intensities obtained in the  
616 presence of methanol were divided by the log<sub>2</sub> intensities obtained without methanol.  
617 These ratios were then normalized by the log median intensity. Genes whose  
618 expression level differed by a factor of 2 or more between the two conditions were  
619 selected for further analysis. Gene ontology analyses were performed using Ecocyc  
620 (<https://ecocyc.org/>). Gene expression data have been deposited in the ArrayExpress  
621 database at EMBL-EBI ([www.ebi.ac.uk/arrayexpress](http://www.ebi.ac.uk/arrayexpress)) under accession number E-  
622 MTAB-8909.

623

#### 624 ***4.8 In silico analysis of methanol metabolism***

625 We used the functions “flux balance analysis” (FBA) and “flux variability analysis” of  
626 the R environment (R Development Core Team, 2009; Team, 2015) Sybil Package  
627 (Gelius-Dietrich et al., 2013) and the genome scale model of *E. coli* ij01366 (Orth et  
628 al., 2011) amended with the heterologous reactions catalyzed by Mdh, Das and Glpx  
629 , and their associated metabolites to simulate the growth and fate of methanol. The  
630 objective function was the growth rate whereas the model was constrained using the  
631 methanol uptake rate measured experimentally for the wild-type methylotroph  
632 *Methylobacter extorquens* (15 mmol/gW/h).

#### 633 **4.9 Growth and methanol consumption calculations**

634 Specific growth rates, uptake rates and production rates were determined using  
635 PhysioFit, provided open source at <https://github.com/MetaSys-LISBP/PhysioFit>. A  
636 conversion factor of 0.37 g dry weight/OD600 was used.

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### 647 **6. Author contribution**

648 A. De Simone, C.M. Vicente and C. Peiro built the strains and performed the  
649 physiological experiments. L. Gales and F. Bellvert performed the MS analysis. B.

650 Enjalbert performed the transcriptomic analysis. S. Heux designed the study and  
651 wrote the paper with the help of all the co-authors. The authors declare that they  
652 have no conflicts of interest.

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## 821 **8. Supplementary Materials**

822 As noted in the text, supplementary materials are available in the online version of  
823 this paper. Supplementary Figures contains Figures S1 to S8. Figure S1 shows and  
824 overview of the recycling of the C1 acceptor Xu5P and its operation. Figure S2 shows  
825 the unrooted phylogenetic trees of selected Mdh and Das homologues. Figure S3  
826 shows the overall scheme of the combinatorial assembly and screening of the  
827 synthetic pathway. Figure S4 shows the expression analysis of *B. methanolicus* Mdh  
828 and *P. angusta* Das in different conditions. Figure S5 shows the western Blot  
829 analysis of expression of Mdh and Das homologues. Figure S6 shows the <sup>13</sup>C-  
830 Methanol assimilation in the methylotrophic *E. coli*  $\Delta$ *frmA* expressing the synthetic  
831 pathway from one or two vectors. Figure S7 shows the <sup>13</sup>C-Methanol assimilation into  
832 central metabolism intermediates in the control strains of the genealogy of  
833 methylotrophic *E. coli*. Figure S8 shows <sup>13</sup>C-Methanol assimilation into proteinogenic  
834 amino acids in the genealogy of methylotrophic *E. coli*.

835 Supplementary Tables contains Tables S1 to S5. Table S1 is the list of selected Mdh  
836 and Das homologues and the associated optimal growth temperature range of the  
837 source organisms. Table S2 contains the mean isotopic enrichment of PEP in %  
838 using the combinatorial library. Table S3 contains the mean isotopic enrichment of  
839 PEP using different Das enzymes with codon-optimized sequences in combination  
840 with MDH from *A. gernerii*. Table S4 contains an overview of the <sup>13</sup>C-enrichment  
841 obtained in different synthetic methylotrophic strains from previous studies. Table S5  
842 is the list of stains and plasmids used in this stu

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# **Mixing and matching methylotrophic enzymes to design a novel “hybrid” metabolic pathway for methanol assimilation in *E. coli***

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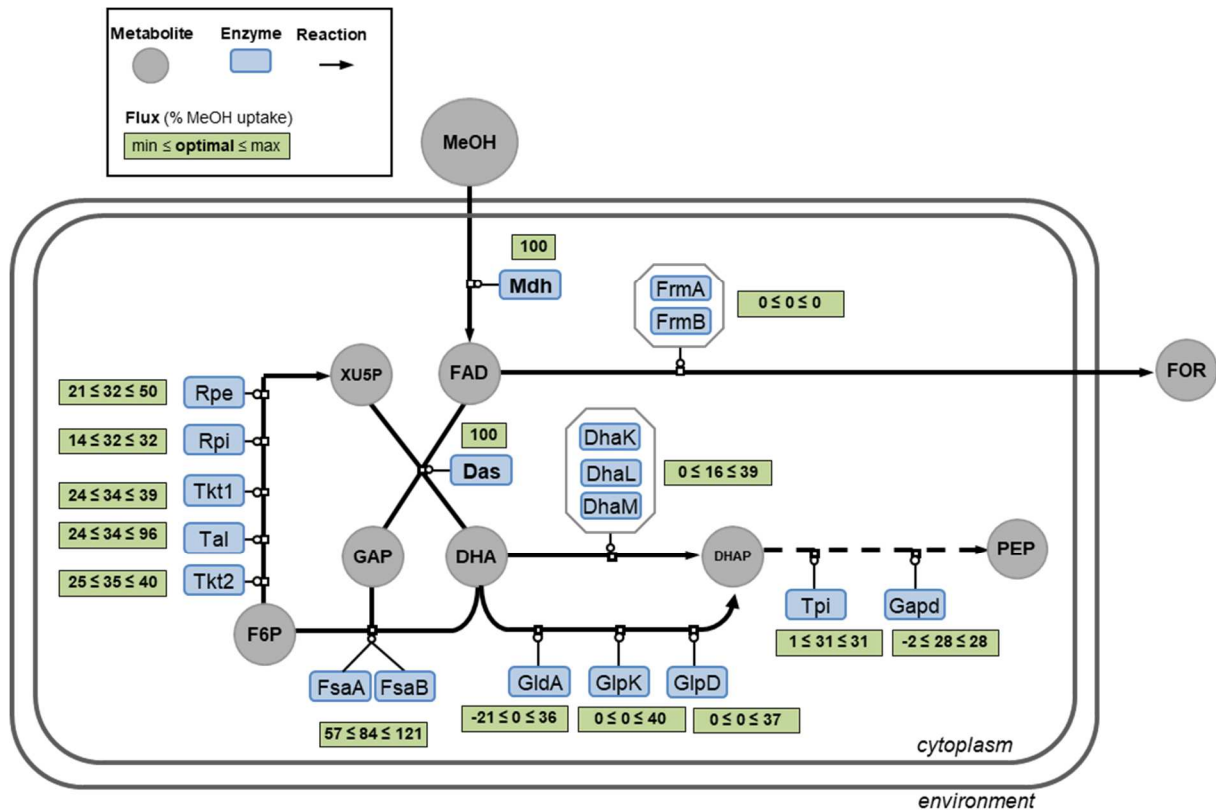
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**Tables 1 and Figures 1 to 4.**

Conditions	Growth rate	Specific xylose uptake rate	Specific formate production rate
+ Methanol	0.17+/-0.00	3.00+/-0.15	0.04+/-0.02
- Methanol	0.11+/-0.01	2.09+/-0.03	0

**Table 1: Physiological response of the new synthetic methylotroph to methanol.** Growth rate ( $\text{h}^{-1}$ ), specific consumption and production rates ( $\text{mmol/g}_{\text{DW}}/\text{h}$ ) of the *E. coli*  $\Delta\text{frmA\_pSEVA424-Mdh-Das}(\text{opt})$  strain during growth in M9 minimal media containing 15 mM xylose without methanol (- Methanol) and supplemented with 150 mM methanol (+ Methanol). Mean and standard deviation of two replicates are given.

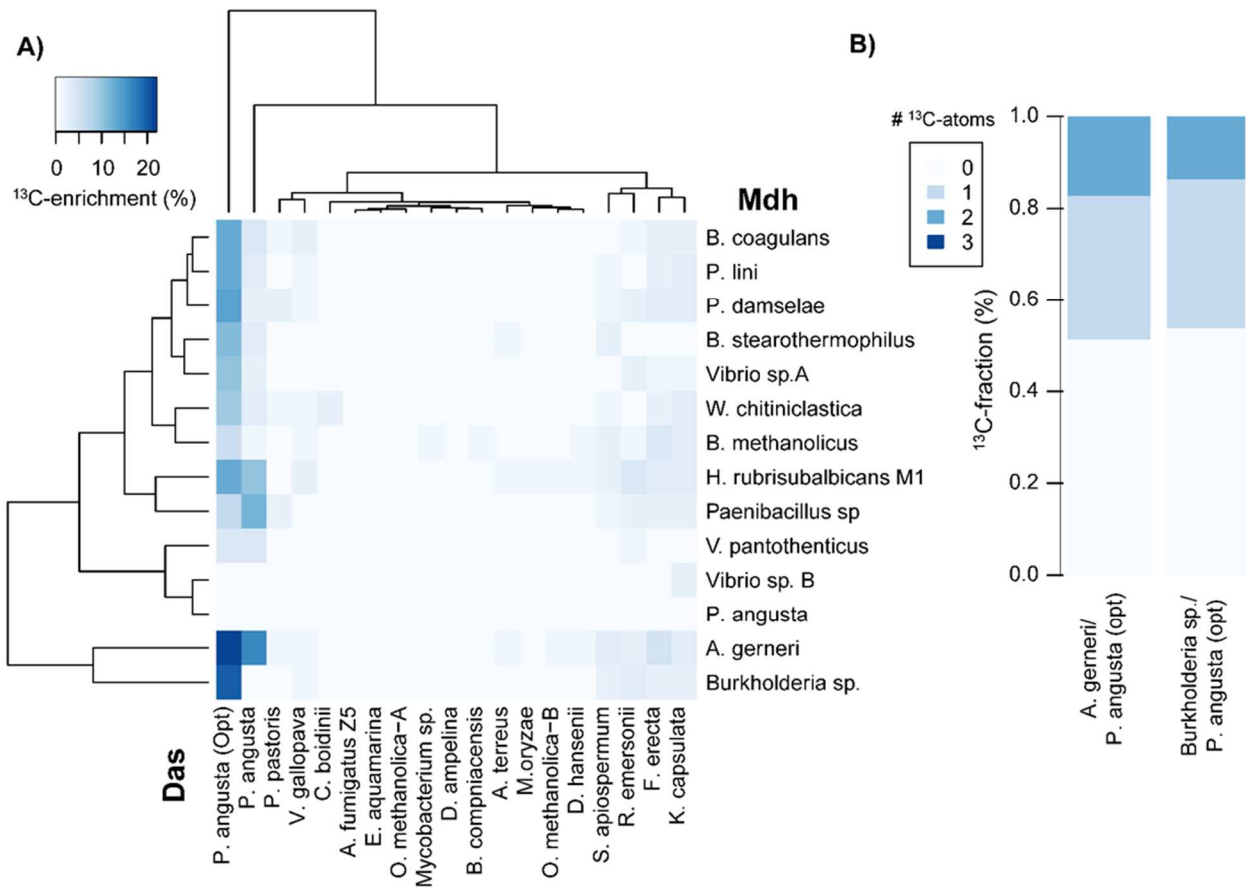




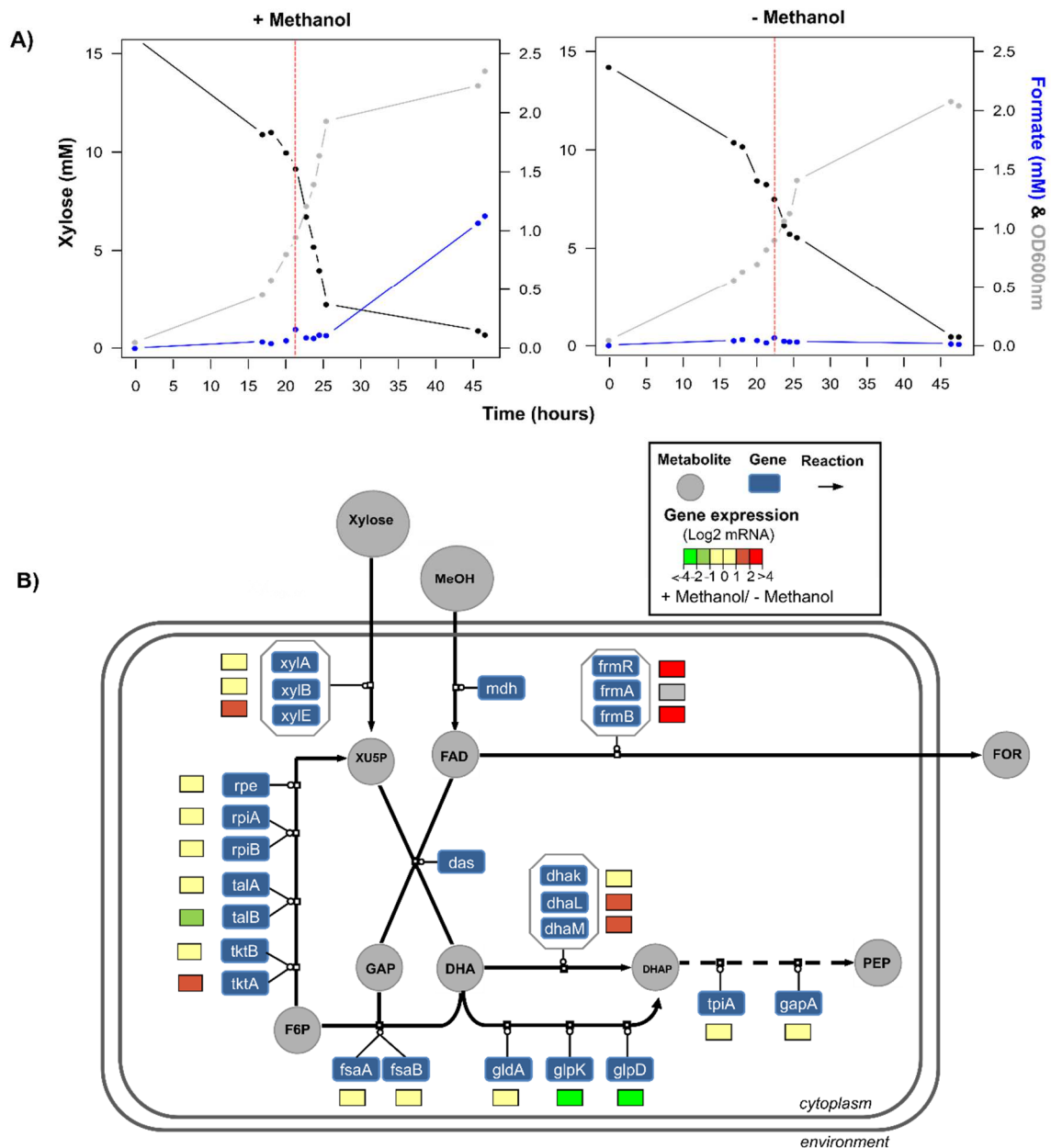
**Figure 1. Overview of the synthetic methanol metabolism and its operation in *E. coli*.**

The new hybrid methanol assimilation pathway comprises a methanol dehydrogenase (Mdh) and a dihydroxyacetone synthase (Das). Green rectangles give the optimal and the ranges of simulated fluxes obtained using flux balance analysis and flux variability analysis, respectively, when growth rate is constrained to 90% of the optimal value. Flux values are given in % relative to a MeOH uptake rate of 15 mmol/gDW/h as defined in Peyraud et al., BMC Syst Biol. 2011.

Dihydroxyacetone kinase (DhaK, DhaL and DhaM); Glycerol dehydrogenase (GldA); Glycerol-3-phosphate dehydrogenase (GlpD); Glycerol kinase (GlpK); Fructose-6-phosphate aldolase (FsaA and FsaB); Triose phosphate isomerase (Tpi); Glyceraldehyde-3-phosphate dehydrogenase (Gapd); Ribulose 5-phosphate 3-epimerase (Rpe); Transketolase (Tkt1 & Tkt2); Transaldolase (Tal), Ribose-5-phosphate isomerase (Rpi); Formaldehyde dehydrogenase (FrmA); S-Formylglutathione hydrolase (FrmB); Methanol (MeOH); Formaldehyde (FAD); Xylulose-5-P (XU5P); Glyceraldehyde-3-phosphate (GAP); Dihydroxyacetone (DHA); Phosphoenolpyruvate (PEP); Dihydroxyacetone phosphate (DHAP); Fructose-6-phosphate (F6P), Formate (FOR).



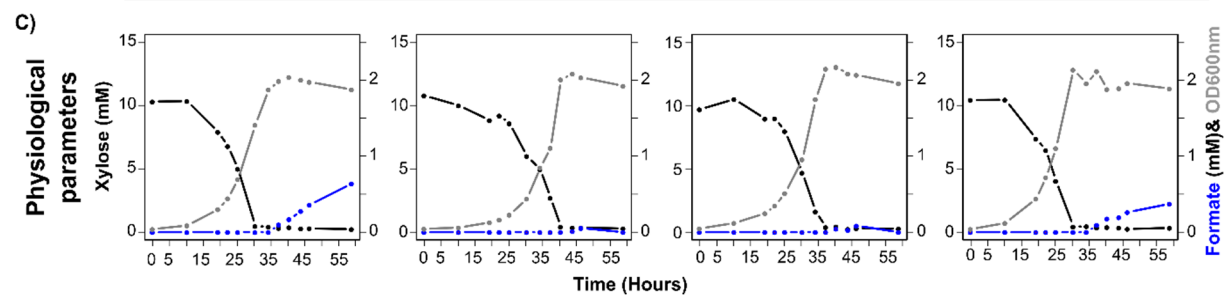
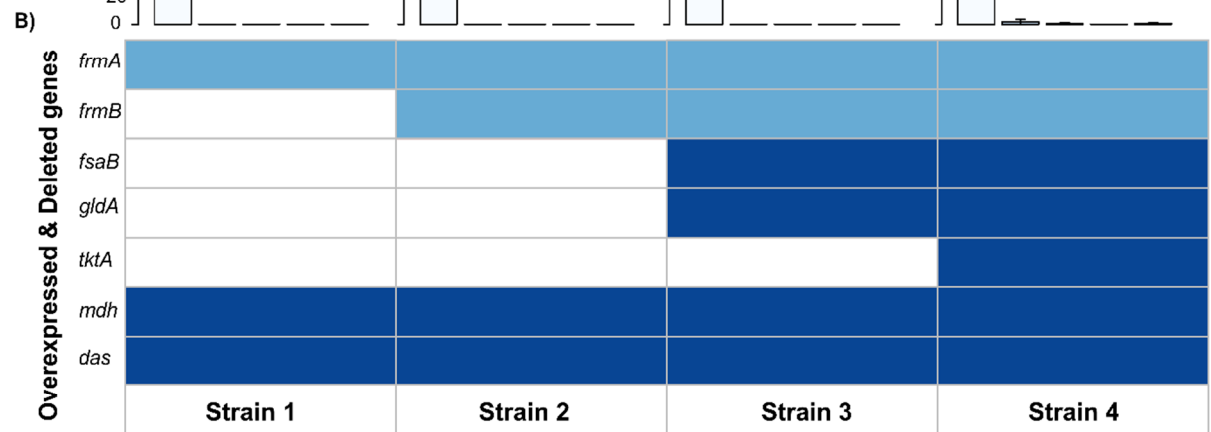
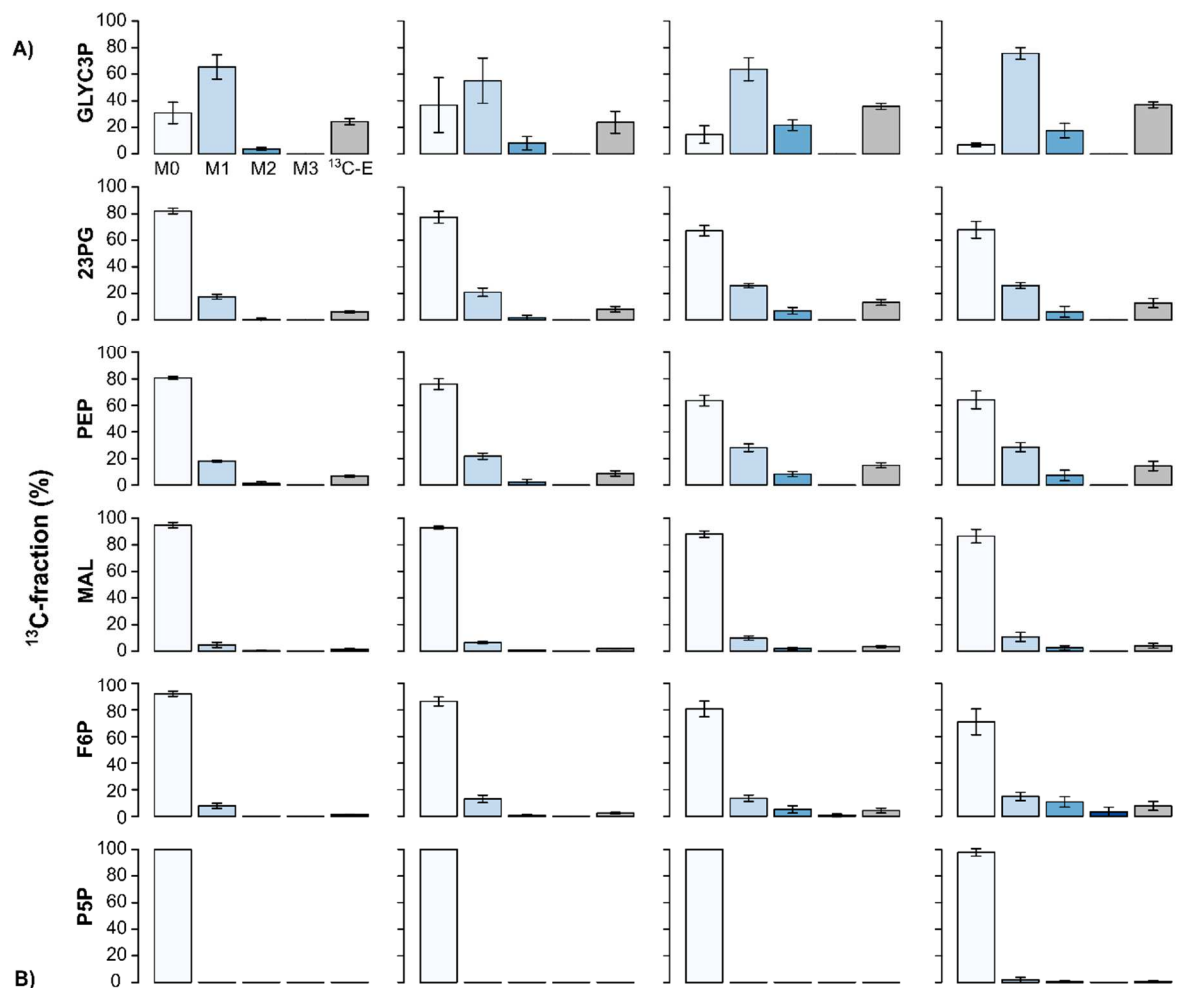
**Figure 2. Screening of the different combinations of the hybrid methylotrophic pathway.** (A) Heatmap showing the  $^{13}\text{C}$ -enrichment of phosphoenolpyruvate (PEP) in *E. coli*  $\Delta\text{frmA}$  expressing different combinations of Mdh and Das homologues. The  $^{13}\text{C}$ -enrichment of PEP was measured at the steady state during exponential growth (90 min after cultivation in M9 medium containing 655 mM of  $^{13}\text{C}$ -methanol). Rows and columns are ordered according to the cluster trees shown on the left and on the top. The Euclidean function was used as distance metric and complete linkage was used as clustering algorithm. (B) Labeling pattern of PEP at 90 min in *E. coli*  $\Delta\text{frmA}$  expressing *P. angusta* Das (opt) with either *A. generi* Mdh or *Burkholderia* Mdh.



**Figure 3. Response of the new synthetic methylotroph to methanol. A)** Time course analysis of xylose (black), formate (blue) and biomass (gray). Red dotted lines indicates the sampling point for transcriptomic analysis. Cells were grown in a minimal synthetic medium containing 15mM xylose with or without 150 mM methanol at 30°C and 250 rpm. One representative experiment for each condition is shown (n=2). **B)** Gene expression profiling of the *E. coli*  $\Delta$ *frmA*\_pSEVA424-Mdh-Das (opt). The coloured squares represent the log<sub>2</sub>-ratios as measure of gene expression fold changes (+ Methanol / - Methanol) during exponential growth at OD<sub>600</sub> = 1 (T1).

Methanol dehydrogenase (*mdh*); Dihydroxyacetone synthase (*das*); Glutathione-dependent formaldehyde detoxification operon (*frmRAB*); Dihydroxyacetone kinase operon (*dhaKLM*); Fructose-6-phosphate aldolase isoform A and B (*fsaA*, *fsaB*); Glycerol dehydrogenase (*gldA*); glycerol kinase (*glpK*); Glycerol-3-phosphate dehydrogenase (*glpD*); Transketolase isoforms A and B (*tktA*, *tktB*); Transaldolase isoforms A & B (*talA*, *talB*); Ribose phosphate isomerase isoforms A & B (*rpiA*, *rpiB*);

Ribulose phosphate epimerase (*rpe*); Triose phosphate isomerase (*tpiA*); Glyceraldehyde 3-phosphate dehydrogenase (*gapA*); Xylose isomerase (*xyIA*); Xylulokinase (*xyIB*); D-xylose/proton symporter (*xyIE*); Methanol (MeOH); Formaldehyde (FAD); Xylulose-5-P (XU5P); Glyceraldehyde-3-phosphate (GAP); Dihydroxyacetone (DHA); Phosphoenolpyruvate (PEP); Dihydroxyacetone phosphate (DHAP); Fructose-6-phosphate (F6P); Formate (FOR).



**Figure 4. Phenotypic characterization of rationally designed *E. coli* strains.** **A)** Labeling patterns of the intracellular metabolites 2 & 3 phosphoglycerate (23PG), fructose-6-phosphohate (F6P), glycerol-3-phosphate (GLYC3P), pool of pentoses-5-phosphate (P5P), phosphoenolpyruvate (PEP) and malate (Mal) within the different strains after 90 min of culture in M9 medium with 655 mM  $^{13}\text{C}$ -methanol. Mean and standard deviation of of 3 replicates are shown. Fraction with zero (M0), one (M1), two (M2) , three (M3)  $^{13}\text{C}$ -atom and  $^{13}\text{C}$ -enrichment ( $^{13}\text{C}$ -E) are show. **B)** Table of overexpressed (dark blue) and deleted (light blue) genes within the different strains. **C)** Time course analysis of xylose (black), formate (blue) and biomass (gray) within the different strains. Strains were grown in a minimal synthetic medium with 15 mM xylose and 150 mM  $^{13}\text{C}$ -methanol at 30°C and 250 rpm. One representative experiment for each strain is shown (n=2).