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# Proteomics of FACS-sorted heterogeneous *Corynebacterium* *glutamicum* populations

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8 **Proteomics of FACS-sorted heterogeneous *Corynebacterium***  
9 ***glutamicum* populations**

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31 **Keywords:** *Corynebacterium glutamicum*, BCAA producer, cellular heterogeneity, FACS,  
32 proteome analysis, label-free quantification

33 **ABSTRACT**

34 The metabolic status of individual cells in microbial cultures can differ, being relevant for  
35 biotechnology, environmental and medical microbiology. However, it is hardly understood in  
36 molecular detail due to limitations of current analytical tools. Here, we demonstrate that FACS  
37 in combination with proteomics can be used to sort and analyze cell populations based on  
38 their metabolic state. A previously established GFP reporter system was used to detect and  
39 sort single *Corynebacterium glutamicum* cells based on the concentration of branched chain  
40 amino acids (BCAA) using FACS. A proteomics workflow optimized for small cell numbers was  
41 used to quantitatively compare proteomes of a  $\Delta aceE$  mutant, lacking functional pyruvate  
42 dehydrogenase (PD), and the wild type. About 800 proteins could be quantified from  
43 1,000,000 cells. In the  $\Delta aceE$  mutant BCAA production was coordinated with upregulation of  
44 the glyoxylate cycle and TCA cycle to counter the lack of acetyl CoA resulting from the deletion  
45 of *aceE*.

46

47

48	<b>ABBREVIATIONS</b>
49	
50	Branched chain amino acids (BCAA)
51	Peptide spectra matches (PSM)
52	Top 3 protein quantification (T3PQ)
53	Leucine responsive protein (LRP)
54	Fluorescence activated cell sorting (FACS)
55	Tricarboxylic acid cycle (TCA)
56	Pentose Phosphate pathway (PPP)

57 **INTRODUCTION**

58

59 **The metabolic status of individual cells in microbial cultures can differ**, and of particular  
60 interest for biotechnology are screening methods for phenotypes where productivity  
61 increases or inadvertently decreases. Cell-sorting methods in combination with proteomics  
62 could then be used to analyze the molecular background of this phenomenon. A requirement  
63 is a method to determine the concentration of a metabolite on the single cell level, for  
64 instance by fluorophore-staining [1] or GFP-reporter systems [2]. To pursue single cell analysis  
65 several techniques, such as flow cytometry, microfluidic chips and single cell genomics, were  
66 developed [3]. Most prominent is flow cytometry, where a directed laminar flow contains cells  
67 in small droplets which are aligned in a pearl chain-like manner. The liquid droplets pass  
68 through detectors which record the characteristics of each single cell. Cytomics is defined as  
69 multimolecular cytometric analysis combined with exhaustive information extraction from all  
70 measured cells [4]. Proteomic analysis of cell samples sorted by flow cytometry can thus be  
71 seen as a domain of cytomics. Proteomics provides an accurate and sensitive approach for  
72 comprehensive protein identification and monitoring of the physiological state of sorted cells.  
73 A first study combining flow cytometry and proteomics analyzed sorted subpopulations of  
74 *C. necator*, formed due to exposure to phenol, with 2D gels [5]. Furthermore, flow cytometry  
75 and MS were combined by using a filter based sample preparation method; here loss of cells  
76 was minimized by using the same filter membrane for storage and digestion. As proof of  
77 principle *P. putida* and *E. coli* K12 cells were mixed and then sorted using flow cytometry and  
78 proteins sequentially identified by MS [6]. For satisfactory proteome coverage about  $5 \times 10^6$   
79 bacteria were required.

80

81 **The bacterium *Corynebacterium glutamicum* is a member of the family of actinomycetales**  
82 **and dominates industrial scale production of amino acids** [7]. The production volumes range  
83 from 2.5 million tons of L-glutamate to 1.5 million tons of L-lysine per year. Also large amounts  
84 of the amino acid L-threonine and of the branched chain amino acids (BCAA) L-leucine and L-  
85 valine are produced [8]. For BCAA production a reporter system was constructed by combining  
86 *eyfp* with a *brnF* promoter, which allows to detect BCAA concentration on the single cell level:  
87 The BrnFE permease exports BCAA and is transcriptionally controlled by the Lrp protein which

88 is activated by binding of BCAA [9]. Increased levels of BCAA lead to expression of eYFP  
89 allowing effective sorting of cells with high BCAA concentrations [2].

90

91 **Engineering efforts leading to increased L-valine production in *C. glutamicum* were centered**  
92 **on the deletion of the pyruvate dehydrogenase complex (PDHC) [10].** BCAA- producing  
93 strains are based on deletion of the *ΔaceE* gene coding for the PDHC subunit E1p leading to  
94 an increased accumulation of pyruvate [11]. Flux analysis of the *ΔaceE* strain already partially  
95 elucidated the carbon flows leading to an increased BCAA production [12], still the protein  
96 networks enabling increased BCAA production must be uncovered.

97

98 In this study, a method was developed to separate cells based on their content of a desired  
99 metabolite and to disclose differences in their metabolic networks by proteomics. The method  
100 was evaluated by characterizing differential abundance of proteins from a mixture of a BCAA-  
101 producing strain and a nonproducing wildtype of *C. glutamicum* cells. Comparison of  
102 proteomes allowed uncovering mechanisms that explain the differences in metabolite  
103 content and enable increased BCAA production.

104 **MATERIALS and METHODS**

105 ***C. glutamicum* strains, media and culture conditions**

106 *C. glutamicum* ATCC 13032 was used as a wild type strain [13], the  $\Delta aceE$  mutant ([14];[10])  
107 was used as BCAA production strain. A first pre-culture of *C. glutamicum* was inoculated with  
108 colonies from a fresh BHI agar plate (brain heart infusion, Difco™ BHI, BD, Heidelberg,  
109 Germany) and grown in 5 ml BHI liquid medium supplemented with 0.5% potassium acetate  
110 for 8 hours at a temperature of 30 °C and a shaking rate of 170 rpm. Afterwards, cells were  
111 washed with 0.9 % (w/v) NaCl and were transferred to 50 ml CGXII minimal medium with 4 %  
112 (w/v) glucose and 1.5 % potassium acetate [15]. The culture for comparison of the  $\Delta aceE$   
113 sensor strain and the WT was inoculated to an OD<sub>600</sub> of 1.0 in this medium and *C. glutamicum*  
114 was grown at 30 °C for 48 hours in 500 ml shaking flasks in 50 ml medium. Cells were cultured  
115 overnight at 30 °C with a shaking rate of 120 rpm.

116

117 Table of Bacterial strains used in this work

Strains	Characteristics	Reference
<i>C. glutamicum</i> ATCC13032	<i>C. glutamicum</i> wild type (ATCC13032) and with chromosomally integrated Lrp sensor (integrated between cg1121-cg1122).	[13]
<i>C. glutamicum</i> ATCC 13032 $\Delta aceE$ sensor strain	<i>C. glutamicum</i> wild type with deletion of the E1p gene ( <i>aceE</i> ) of the PDHC and with chromosomally integrated Lrp sensor (integrated between cg1121-cg1122).	[2]

118

119

120 **Flow cytometry measurements**

121 Flow cytometry measurements were performed using a FACS Aria II cell sorter (Becton-  
122 Dickinson, Heidelberg, Germany) using a blue solid state laser (Sapphire™ 488-20) with an  
123 excitation wavelength of 488 nm and a power of 13 mW. Cytometer setup and performance  
124 tracking was performed with Cytometer Setup & Tracking Beads (bright (3 μm), mid (3 μm),  
125 and dim (2 μm) beads) labeled with a mixture of fluorochromes (Becton Dickinson, Heidelberg,  
126 Germany). EYFP fluorescence was detected via a 502-nm long pass and a 530/30-nm band-  
127 pass filter set. Data were recorded with the FACS Diva software 6.0 and were analyzed with  
128 the FlowJo flow cytometry analysis software 7.6.5 (Tree Star, Ashland, USA).

129



130 **Cell sorting procedure**

131 Cell sorting was performed in the four-way purity precision mode with flow rates  $\leq 3$  using the  
132 FACSria II cell sorter. Drop delay was set with FACS™ Accudrop Beads (Becton Dickinson,  
133 Heidelberg, Germany) containing a single population of 6- $\mu\text{m}$  particles that consist of a  
134 fluorophore that is excited at 670 nm and emits at 750 nm. Cells were sorted and collected on  
135 a 96-well plate containing a hydrophilic polyvinylidene fluoride (PVDF) membrane at the  
136 bottom (Millipore, Schwalbach, Germany) to an amount between  $1 \times 10^4$  and  $1 \times 10^6$  cells per  
137 filter membrane. The multi-dish plate was connected to a vacuum pump so that the buffer  
138 could be directly removed and cells could be concentrated on the filter membranes. Thereby,  
139 three replicates for each sample were performed. Filter membranes could be stored at  $-20^\circ\text{C}$   
140 until cell disruption.

141

142 **Cell lysis and protein digestion**

143 Filter membranes containing bacterial cells were divided into smaller pieces and were  
144 dissolved in 32  $\mu\text{l}$  dissolution buffer (25 mM ammonium bicarbonate, pH 7.8 containing  
145 2  $\mu\text{l}$  acetonitrile). Subsequently, cells were proteolytically digested with 8  $\mu\text{l}$  trypsin (Promega,  
146 Mannheim, Germany) resulting in a working concentration of 0.25  $\mu\text{g}/\mu\text{L}$  at  $37^\circ\text{C}$  with  
147 continuous shaking at 400 rpm for 2 hours. Afterwards, cell debris and filter membranes were  
148 removed by centrifugation at 13,000 g for 10 minutes at RT. Supernatants were collected in a  
149 new tube and were stored up to one week at  $-20^\circ\text{C}$ .

150

151 **Protein identification via 1D-nLC-ESI/MS**

152 The lyophilized tryptic digests were re-suspended in buffer A (0.1 % v/v formic acid in water)  
153 by ultrasonication and subjected to mass spectrometric analyses, which were performed using  
154 a nanoAcquity UltraPerformance LC System connected to an auto-sampler equipped with a  
155 HSS T3 analytical column (1.8  $\mu\text{m}$  particle, 75  $\mu\text{m}$  x 150 mm) kept at  $45^\circ\text{C}$ , and a Symmetry C18  
156 trap column (5  $\mu\text{m}$  particle, 180  $\mu\text{m}$  x 20 mm) (all Waters, USA) as well as a PicoTip Emitter  
157 (SilicaTip, 10  $\mu\text{m}$ ) from New Objective (USA) as a nanospray source, coupled to an LTQ  
158 Orbitrap Elite mass spectrometer from Thermo Fisher Scientific Inc. (USA). The LTQ Orbitrap  
159 was operated by instrument method files of Xcalibur (Rev. 2.2 SP1). The linear ion trap and  
160 Orbitrap were operated in sequence, i.e. after a full MS scan on the Orbitrap in the range of  
161 300-2000 m/z at a resolution of 60,000, the 10 most intense precursors were subjected to CID

162 fragmentation (ion target value of 10,000, activation time of 10 ms, 400ms maximal inject  
163 time, 35 % normalized collision energy) and fragments detected in the ion trap. The heated  
164 desolvation capillary was set to 275 °C. Dynamic exclusion was enabled with a repeat count of  
165 1 and a 45 sec exclusion duration window. Singly charged ions and ions of unknown charge  
166 state were rejected from MS/MS. Flow rate was set to 400 nl/min and spray voltage was set  
167 to 1.5-1.8 kV. Peptides were eluted from Trap column onto a separation column using a multi-  
168 step gradient of buffer A to buffer B (0.1 % v/v formic acid in acetonitrile). A 180 min gradient  
169 was used: (0-5 min: 99 % buffer A and 1 % buffer B, 5-10 min 99 %-94 % A, 10-161 min: 94 %-  
170 60 % A, 161-161.5 min: 60 %-14 % A, 161.5-166.5 min: 14 %-4 % A, 166.5-167.1 min: 99 % A,  
171 167.1 min-180 min: 99 % A).

172

### 173 **Database searches**

174 All database searches were performed using SEQUEST algorithm, embedded in Proteome  
175 Discoverer™ (Rev. 1.3, Thermo Electron © 1998-2007), with a *C. glutamicum* ATCC 13032  
176 database containing 3058 sequences, which was provided by Jörn Kalinowski from Bielefeld  
177 University [13]. Only tryptic peptides with up to 2 missed cleavages were accepted. No fixed  
178 modifications were considered. Oxidation of methionine was permitted as variable  
179 modification. The mass tolerance for precursor ions was set to 10 ppm; the mass tolerance for  
180 fragment ions was set to 0.8 amu. For search result filtering, a peptide FDR threshold of 0.01  
181 (q-value) according to Percolator was set in Proteome Discoverer, and at least two unique  
182 peptides with search result rank 1 were required.

183

### 184 **Label-free quantification**

185 For Top 3 Protein Quantification (T3PQ) ([17]; [18]), the average area of the three unique  
186 peptides of a protein with the largest peak area was calculated by Proteome Discoverer. This  
187 quantification method was used to obtain the area values for the data presented here. The  
188 mass spectrometry proteomics data have been deposited to the ProteomeXchange  
189 Consortium via the PRIDE [1] partner repository with the dataset identifier PXD005812 and  
190 10.6019/PXD005812.

191

192

### 193 **Bioinformatics**

194 Samples were standardized based on a z-score normalization, i.e. for each sample and  
195 replicate the respective mean value and standard deviation was calculated and used to  
196 normalize each measurement. Afterwards an ANOVA was calculated comparing all replicate  
197 measurements of sample P1 against sample P2. Aiming at an utmost comprehensive set of  
198 potentially interesting candidate proteins, an adjustment of p-values to compensate for the  
199 multiple testing situation has deliberately been omitted. Principal component analyses were  
200 performed comparing P1 and P2. At this only proteins with complete series of measurements  
201 were taken into consideration with replicate measurements being combined by calculating  
202 their mean value. All analyses were carried out in the R environment for statistical computing  
203 and graphics using standard packages (“stats”, “graphics”) [19].

204

205

206 **RESULTS**

207  
208 **Workflow for small cell number proteomics with FACS-sorted *C. glutamicum***

209  
210 **Protein Identification in small cell number samples**

211  
212 **A robust, yet sensitive proteomics workflow is required for protein identification in small**  
213 **cell number samples or subpopulations acquired by cell sorting.** The low protein content of  
214 single *C. glutamicum* cells in the range of 0.13 pg (own unpublished result) to 0.2 pg (calculated  
215 for *E. coli* from [20]) makes it obvious that a very sensitive proteomics method has to be used.  
216 The filter-based cell disruption approach minimizes loss of cells and allows for sequential  
217 sample preparation in the same environment and can be combined with different MS  
218 proteomics setups [6]. This method was used to establish the correlation of sorted cells to  
219 protein identifications by sorting 1,000,000, 100,000 and 10,000 *C. glutamicum* WT cells and  
220 subsequent preparation. From 1,000,000 cells (about 130 ng) 489 proteins were identified,  
221 100,000 cells led to identification of 107 proteins and 10,000 cells led to identification of 61  
222 proteins (Fig. 1). As the discrepancy between 1,000,000 cells and lower cell numbers was too  
223 large, optimization of MS methods was only performed for 1,000,000 cells, where  
224 identification was improved by increasing the filling time from 150 to 400 ms to better account  
225 for the low amount of sample loaded. This optimization led to protein identification rates on  
226 average close to 650 proteins.

227  
228 **Identified proteins were quantified using spectral counting and T3PQ.** To assess correlation  
229 of quantification results between both methods principal component analysis was performed  
230 for a combined dataset from several experiments. Principal components were determined and  
231 used to represent the covariance in the z-standardized dataset. For PSM and T3PQ  
232 quantification similar variation between experiments was observed for the first and second  
233 principal components (data not shown). Based on these results we decided to preferably use  
234 T3PQ quantification for presenting our data in the following experiments.

235  
236 **Proteomic evaluation of FACS sorting based on the metabolic state of *C. glutamicum* cells.**  
237 Cytometric analysis shows that cells of *C. glutamicum* WT strain and  $\Delta aceE$  mutant exhibited

238 different levels of eYFP fluorescence. For the FACS sorting experiment cells were sampled after  
239 12 hours when increased eYFP fluorescence generated by the Lrp sensor is detectable in L-  
240 valine producing *ΔaceE* strains. Active fluorescence caused by the Lrp sensor is required to  
241 distinguish BCAA-producing cells from cells not producing BCAA. To demonstrate that this  
242 workflow can successfully be applied to uncover proteome differences *ΔaceE* and WT strain  
243 cells were sorted from separate cultures first. For the eYFP fluorescence most cells from the  
244 *ΔaceE* strain show fluorescence intensity between  $10^3$  and  $10^5$ , while most WT cells only  
245 exhibit fluorescence levels below  $10^2$  (**Fig. 2 b**). Of note, the cells of the WT and *ΔaceE* strain  
246 do not show a distinction in cell size and cell morphology as the FSC-A readout between both  
247 populations demonstrates. Therefore, the main distinction between these cells is not a change  
248 in morphology but the physiological changes leading to increased BCAA production. The  
249 reliability of the *ΔaceE* cell sorting procedure applied here was established in a previous study  
250 by sorting *ΔaceE* single cells onto agar plates from a mixture with *C. glutamicum* WT only  
251 containing 1% *ΔaceE* strain cells. Strains were distinguished by the small colony phenotype of  
252 *ΔaceE* and subsequent colony PCR showing a 95% correct sorting efficiency for *ΔaceE* [2]. The  
253 sorted cells were subjected to the filter based sample preparation approach for proteome  
254 analysis.

255  
256 **Principal component analysis of quantitative proteome data was performed to assess, if the**  
257 **proteome of *ΔaceE* and WT cells sorted from mixed cells shows the same features as *ΔaceE***  
258 **and WT proteomes extracted from separate cultures (Fig, 2c.) to validate FACS sorting.** For  
259 the first (**Comp. 1**) and second principal component (**Comp 2**) the proteome of *ΔaceE* cells  
260 sorted from the mixed culture displayed an almost identical orientation of its dataset to the  
261 *ΔaceE* dataset from separate cultures. Additionally, ANOVA was done to find individual  
262 proteins that may differ in their concentration between samples from mixed and separate  
263 cultures of the respective strains (Supp. Table 1). Only 97 of 728 identified proteins showed a  
264 p-value below 0.05 and thus differed in amount between mixed and separated samples. Still  
265 these proteins did not change significantly according to the adjusted p-value. Based on PCA  
266 and ANOVA, it can be concluded that the proteome of the WT cells sorted from the mixed  
267 culture and the WT cells from the separate cultures behaved in the same fashion. Hence, FACS  
268 could successfully separate cells based on metabolite content and mixing of the two cultures

269 did not affect the proteome. **Characteristic differences between WT and  $\Delta aceE$  strain**  
270 **proteomes**

271

272 **It was our intention to demonstrate that the small cell numbers obtained by FACS**  
273 **separation allow for comprehensive proteome comparison of *C. glutamicum*.** Here, this  
274 would allow characterizing physiological features of BCAA production in *C. glutamicum*  $\Delta aceE$   
275 in comparison to no production in *C. glutamicum* WT, both cultivated for 12 hours. After FACS,  
276 in total 960 proteins were identified and quantified across all replicates from separate  
277 samples. 701 of these proteins were identified in the  $\Delta aceE$  strain as well as in the WT strain.  
278 Only 56 proteins were exclusive to the WT strain and 203 proteins to the  $\Delta aceE$  strain. Label  
279 free quantification of the identified proteins enables interpretation of the changes in  
280 metabolic pathways given in detail below (Table 1).

281

282 **In the mutant the glycolysis pathway is utilized as a main energy source, also providing**  
283 **precursors for the production of BCAA.** Unsurprisingly, the largest decrease of an enzyme in  
284 the mutant was reported for the AceE subunit of the pyruvate dehydrogenase complex with  
285 a complete disappearance of the enzyme. Glycolytic enzymes as glyceraldehyde-3-phosphate  
286 dehydrogenase (GAP) and enolase showed decreased abundances in the  $\Delta aceE$  mutant,  
287 reflected in a regulation factor of -0.15 and -0.33, respectively. To increase carbon supply for  
288 glycolysis the phosphoglycerate dehydrogenase, an enzyme directing carbon away from the  
289 glycolytic pathway, was downregulated in the  $\Delta aceE$  mutant by -0.41.

290

291 **Deletion of the pyruvate dehydrogenase is accompanied by upregulation of the TCA cycle**  
292 **and the glyoxylate cycle.** Disappearance of the pyruvate dehydrogenase complex leads to a  
293 decline of acetyl-CoA levels in the  $\Delta aceE$  mutant (Bartek et al. 2011). Enzymes of the TCA also  
294 being part of the glyoxylate cycle were upregulated in the  $\Delta aceE$  strain (Fig. 3). Based on the  
295 regulation factors for citrate synthase (CS), aconitase (ACO), isocitrate dehydrogenase (IDH)  
296 and succinyl CoA synthetase (SUC) an increase in abundance in  $\Delta aceE$  was found. The only  
297 enzymes with a decrease in the mutant were malate dehydrogenase (MDH) and fumarate  
298 hydratase (FUM) both having a regulation factor of -0.39, awhile the counterpart of MDH for  
299 this reaction malate quinone oxidase (MQO) was slightly upregulated (0.24). Glyoxylate cycle  
300 enzymes show strong overexpression in the  $\Delta aceE$  mutant, especially isocitrate lyase (IL) with

301 a regulation factor of 1.13 strongly increased in the mutant. Malate synthase (MS) too was  
302 upregulated as shown by the regulation factor of 0.53. Upregulation of these enzymes is  
303 similar to that of the acetate fixation pathway where acetate kinase and phosphate  
304 acetyltransferase as well as the succinyl acetate CoA transferase (SCoA) were upregulated.  
305 Considerable upregulation occurred for the pyruvate carboxylase reaction, which replenishes  
306 oxaloacetate from pyruvate while consuming one molecule of ATP, too. Apparently, in  
307 response to absence of the pyruvate dehydrogenase complex reaction the TCA was provided  
308 with carbon from replenishing pathways which consume energy and provide less NADH.  
309  
310

311 **DISCUSSION**

312  
313 **Workflow for small cell number proteomics with FACS-sorted *C. glutamicum***

314  
315 **A prerequisite for successfully performing proteomics with small cell numbers is a workflow**  
316 **that minimizes sample loss.** The method of Jehmlich et al. [6] was chosen as it allows  
317 combining several sample processing steps, e.g. as lysis and digestion, to take place in one  
318 compartment. Aiming to further optimize the procedure for our experiments, we found that  
319 the use of an increased filling time was the key for improved proteome coverage. The maximal  
320 number of protein identifications our small cell number proteomics method can attain was  
321 calculated as the mean of three 1,000,000 cells samples of the *C. glutamicum* WT proteome  
322 and could be calculated to be 863 proteins, this equals a 28.7% proportion of the total  
323 proteome. In a 2013 study for 5,000,000 *P. putida* KT2440 cells a total of 743 unique proteins  
324 were identified in 4 replicates, equaling a proportion of 13.7% of the global proteome [21].  
325 Combining the sample processing developed by Jehmlich et al. with our mass spectrometry  
326 setup and the improvements in the measurement methods enabled us to identify proteins in  
327 small subpopulations for the first time for the organism *C. glutamicum*. Small cell number  
328 proteomics methods developed for eukaryotes [21] were not tested for our experiments as  
329 the cell wall of prokaryotes shows much higher level of rigidity in comparison to eukaryotes,  
330 which requires a harsher lysis procedure.

331  
332 **Proteomic evaluation of FACS sorting based on the metabolic state of *C. glutamicum*.** In this  
333 study we used an approach that relies on sorting of fluorescent cells containing the LRP sensor  
334 system reporting on BCAA concentrations to assess the changes in the proteome leading to  
335 BCAA production. Fluorescent cells are a prerequisite for FACS sorting, therefore in early  
336 studies DAPI staining of cellular DNA was used to separate *E. coli* and *P. putida* cells displaying  
337 by way of proteomics that this sorting process is very efficient [6]. Furthermore in a 2013 study  
338 *P. putida* cells producing a fluorescent eGFP protein fused to the *styA* gene were sorted based  
339 on eGFP fluorescence and forward scatter. Using the two parameters cells were sorted into a  
340 group showing no fluorescence due to high DNA synthesis, as well as into a group of sorted  
341 fluorescent cells with a high forward scatter exhibiting a high accumulation of the *styA* protein  
342 and decreased cell cycle activity [21]. In contrast to these previous approaches the novelty of



343 our approach lies in sorting and selecting cells based on their metabolic status, here  
344 production or no production of the small molecule BCAA. This enables proteomics for the  
345 direct interrogation of changes in the metabolic pathways and other cellular functions in  
346 producing and non-producing subpopulations.

347

348 **Assessment of FACS sorting efficiency with proteomics.** The LRP reporter sensor system is  
349 known to be very robust in reflecting different levels of BCAA production as well as its specific  
350 fluorescence 2 times stronger than unspecific background fluorescence, hence being a good  
351 marker for BCAA nonproduction using cytometric methods [2]. However, the efficiency of  
352 FACS sorting using such a metabolite concentration reporter system remained to be verified  
353 on the molecular level, as done here for the proteome. For this purpose, the small cell number  
354 proteomes of WT *C. glutamicum* cells and  $\Delta aceE$  cells were analysed from pure cultures and  
355 FACS-separated mixed cells to corroborate differences in physiology indicated by fluorescence  
356 as well as by PCA and ANOVA analysis of the proteomes. The proteomes of BCAA  
357 nonproducing WT from the mixed culture as well as cells sorted from independent cultures  
358 are very similar, same is true for the BCAA producing  $\Delta aceE$  cells. Overall, PCA and ANOVA  
359 validated that the differences in proteomes was larger between strains than between mixed  
360 samples and samples from pure cultures of the same strain. This fits well to the results of a  
361 previous study by [6] where it was shown that *E. coli* K12 and *P. putida* KT2440 cells can be  
362 sorted with high specificity. Thus, FACS can efficiently sort cells without the sorting process  
363 affecting strongly the proteome status. This has already been shown for prokaryotic  
364 proteomes by [6]. Whereas Jehmlich used fixation of the cells, we assumed that our short  
365 sorting time should hardly affect the proteome, hence fixation could be omitted for proteome  
366 studies. Moreover, the insignificant differences between the proteomes from sorted and pure  
367 cultures demonstrate that properly designed eYFP fluorescence reporter systems can  
368 faithfully discriminate the physiological state – at least on the proteome level - in cell mixtures.  
369 This also implies that in addition FACS sorted cells can be used to reliably assess regulation  
370 mechanisms between producing and nonproducing strains or subpopulations.

371

372 **Characteristic differences between WT and  $\Delta aceE$  strain proteomes**

373

374 **C3 and C4 stockpiles in the TCA are replenished using malate provided by the glyoxylate**  
375 **cycle.** ANOVA analysis of the proteomes substantiated that the proteins involved in central  
376 carbon metabolism as well as in amino acid metabolism are significantly regulated towards  
377 the metabolisation of acetate. The enzymes adding most to the variation between  $\Delta aceE$   
378 strain proteome and WT strain proteome are isocitrate lyase (Cg2560), citrate synthase  
379 (Cg0949) and phosphate acetyltransferase (Cg3048). This is in line with previous research,  
380 which showed that in case of removal of precursors from the TCA cycle, this cycle is refilled by  
381 the glyoxylate cycle [22] as happens in the  $\Delta aceE$  strain, thus the TCA and glyoxylate cycle in  
382 the  $\Delta aceE$  strain are upregulated in comparison to the WT in contrast to *E. coli* where the  
383 glyoxylate cycle is repressed when glucose and acetate are both present. This enables parallel  
384 metabolization of acetate and glucose in *C. glutamicum* [22]. A previous study found that  
385 during growth on glucose+acetate the glyoxylate cycle is used to replenish the malate pool  
386 which is needed for the TCA [22]. Also induction of TCA cycle gene transcripts in WT  
387 *C. glutamicum* cells only grown on acetate was reported in 2002 [23]. Under these conditions  
388 acetyl-CoA is predominantly produced from acetate [22]. This is corroborated by metabolic  
389 flux data, as in PDHC deficient strains fed with labeled glucose and unlabeled acetate a large  
390 fraction of unlabeled TCA intermediates persists [12]. Upregulation of enzymes belonging to  
391 the glyoxylate cycle provide C3 and C4 molecules for anabolic reactions in the cells [24].

392  
393 **Upregulation by the RamB transcriptional regulator can be found for several pathways of**  
394 **acetyl-CoA synthesis bypassing the pyruvate dehydrogenase complex.** A genetic mechanism  
395 for the upregulation of the IL and MS genes taking part in the glyoxylate cycle via repression  
396 by RamB has been established [25]. Also RamB binding motifs were computationally identified  
397 in front of the genes for pyruvate carboxylase and citrate synthase. Pyruvate carboxylase as  
398 well provides intermediates for the TCA cycle by synthesizing oxaloacetate from pyruvate. Our  
399 data corroborates concomitant possible induction of pyruvate carboxylase and citrate  
400 synthase by RamB as suggested by computational detection of binding motifs in the *C.*  
401 *glutamicum* WT genome [25]. Previous studies show these proteins are already upregulated  
402 in the WT due to the co-metabolization of acetate and glucose. Still we detect a higher  
403 abundance of these proteins in the  $\Delta aceE$  evidencing an influence of the acetyl-CoA pool on  
404 the level of gene expression controlled by RamB. Another bypass using the up-regulated SCOA,

405 where regulation is unknown, can convert acetate to acetyl-CoA by transfer of the CoA group  
406 from succinyl-CoA [26].

407

408 **The AHAS enzyme, a central step in BCAA synthesis, showed only low upregulation in  $\Delta aceE$ .**

409 This low level upregulation is mirrored by flux data where a low level increase of carbon flux  
410 could be detected when comparing WT and  $\Delta aceE$  [12]. In  $\Delta aceE$  an increased pool of pyruvate  
411 is available [27]. This raised concentration is favourable for the acetohydroxyacid synthase  
412 (AHAS) enzyme which has a high  $K_m$  for pyruvate at 8.3 mM [28]. The synthesis pathways of  
413 leucine, isoleucine and valine are regulated at the AHAS reaction step by feedback inhibition  
414 with BCAA [28].

415

416 **Regulation of enzymes utilizing pentose phosphate pathway and glycolysis for increased**

417 **BCAA synthesis.** The proteome data for the carbon metabolism points to differential  
418 regulation between  $\Delta aceE$  and the WT strain. A decreased glucose consumption has been  
419 documented for the  $\Delta aceE$  strain [12], also we found a slight downregulation of GAPDH and a  
420 stronger decrease of the glycolytic enzyme enolase. Still for the enolase reaction a strong  
421 increase in flux for the  $\Delta aceE$  strain has been measured [12], suggesting allosteric regulation  
422 and/or PTM based regulation as a means to increase the activity of the enolase enzyme. Our  
423 dataset also records only small upregulation for the pentose phosphate pathway enzymes  
424 phosphoglucoconolactonase (Tab. 1), this is in line with the small switch of carbon flows from  
425 glycolysis to the pentose phosphate pathway (PPP) [12]. The strongest impact of the mutation  
426 takes place in pyruvate metabolism as the pyruvate dehydrogenase subunit E1 is deleted in  
427 the  $\Delta aceE$  strain. As a consequence of this pyruvate accumulation takes place and the carbon  
428 from glycolysis only directly enters the TCA through the pyruvate carboxylase reaction.  
429 Downregulation of enolase and phosphoglycerate dehydrogenase as well as deletion of the  
430 *aceE* gene impact the glycolytic pathway as more glucose is converted into pyruvate to feed  
431 the pyruvate carboxylase reaction as well as BCAA biosynthesis.

432

433 Our data provides an insight into fundamental changes of the carbon metabolism in *C.*  
434 *glutamicum* deficient of the pyruvate dehydrogenase function. To counter the lack of acetyl  
435 CoA resulting from the deletion of the PDHC E1 enzyme, BCAA production is coordinated with  
436 upregulation of the glyoxylate cycle and TCA cycle. The inability of the mutant to refill the TCA

437 cycle via pyruvate decarboxylation leads to the uptake of acetate via alternative pathways as  
438 SCOA. For provision of TCA cycle intermediates the glyoxylate cycle and pyruvate carboxylase  
439 pathway are activated. The accumulating pyruvate is converted to BCAA.

440

## 441 **CONCLUSION**

442

443 In this study, it was shown that the combination of FACS and proteomics is suitable for the  
444 selection and molecular characterization of microbial cells differing in the concentration of  
445 metabolites. In conclusion, we could demonstrate that small cell number proteomics is able  
446 to compare a BCAA producing strain and the WT after FACS sorting and exemplify the effect  
447 of deletion of a central step in carbon metabolism on the physiology of *C. glutamicum*. The  
448 used technology could enable proteomic characterization of biotechnologically significant  
449 emergence of a fast growing, non-productive subpopulation.

450

## 451 **ACKNOWLEDGEMENTS**

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453 financial support in the FlexFit initiative (0315589A as well as 0315589E). Furthermore we  
454 thank Julia Frunzke, Regina Mahr and Andrea Neumeyer from Research center Jülich for  
455 provision of samples and help with sample preparation. Also we thank Nils Bardeck for  
456 providing information on the protein content of single *C. glutamicum* cells.

457

458

459 Figures:

460

461 Fig. 1: Identified proteins relating to the applied cell number of *C. glutamicum* ATCC 13032  
462 wild type. 100,000 cells and 1,000,000 cells were measured in two technical replicates, the  
463 means of both replicates being presented, while for 10,000 cells three technical replicates  
464 were measured. In three technical replicates an LC/MS method with an increased filling time  
465 and a higher number of fragmented peptides enabled identification of up to 800 proteins and  
466 a mean protein identification number of 650 proteins was achieved.

467

468

469 Fig. 2: a) Schematic view of experimental procedures for cell selection and sorting.  
470 *C. glutamicum* ATCC13032 as well as a *C. glutamicum*  $\Delta aceE$  with integrated LRP sensor were  
471 grown in CGXII medium containing 4% glucose and 1.5 % potassium acetate. Cells from both  
472 cultures were mixed together 1:1 after 12 hours growth time, then a FACS Aria II cell sorter  
473 was used to identify and sort cells based on the emergence of eYFP fluorescence in the  $\Delta aceE$   
474 cells (green). b) Sorting of cells presented by correlation of forward cell scatter and eYFP  
475 fluorescence for the WT and  $\Delta aceE$  strain. c) Principal component analysis of the *C.*  
476 *glutamicum* ATCC 13032 and *C. glutamicum*  $\Delta aceE$  proteomes. Principal component analysis  
477 was performed for z standardized proteome datasets for *C. glutamicum* ATCC 13032 cells and  
478 *C. glutamicum*  $\Delta aceE$  cells acquired by FACS-sorting from a mixture and from each of the  
479 cultures before mixing. The red arrows indicate the directions of the proteome datasets in  
480 regards to the principal components

481

482

483 Fig. 3: a) Schematic presentation of enzymes (squares) involved in glycolysis and valine (BCAA)  
484 production. Arrows represent enzymes in the *C. glutamicum*  $\Delta aceE$  and the *C. glutamicum*  
485 ATCC 13032 strain. Enzyme names are given in squares. The regulation factor of up- or  
486 downregulation of enzymes in the  $\Delta aceE$  mutant is represented by the color of the squares  
487 adjacent to the enzyme names as indicated in the color scale. Enzymes displayed in this figure  
488 are: HK = hexokinase, TPI = triose phosphate isomerase, GAP = glyceraldehyde-3-phosphate  
489 dehydrogenase, PHGDH = phosphoglycerate dehydrogenase, PGM = phosphoglycerate  
490 mutase, ENO = enolase, PYK = pyruvate kinase, ILVB = acetolactate synthase, DHAD =  
491 dihydroxyacid dehydratase, AceE = pyruvate:dehydrogenase complex. b) Schematic  
492 presentation of enzymes (squares) involved in Tricarboxylic acid cycle (TCA) and the glyoxylate  
493 cycle. Regulation factor of up- or downregulation of enzymes in the  $\Delta aceE$  mutant is  
494 represented by the color of the squares adjacent to the enzyme names as indicated in the  
495 color scale.

496 Enzymes displayed in this figure are: MDH = malate dehydrogenase, CS = citrate synthase, ACO  
497 = aconitase, IDH = isocitrate dehydrogenase, IL = isocitrate lyase, MS = malate synthase, KGD  
498 =  $\alpha$ -ketoglutarate-dehydrogenase, SCOA = succinyl acetate CoA transferase, SUC = succinyl  
499 CoA synthetase, SDH = succinate dehydrogenase, FUM = fumarase.

500

501 Supp. Fig. 1: Volcano Plots calculated for WT and  $\Delta aceE$ . Log(2) logarithmized regulation  
502 factors were plotted against the  $-\log_{10}$  P-values for every protein identified in WT and  $\Delta aceE$ .  
503 Cutoff for significant regulation was set to 0.05.

504

505 Tables:

506

507

508

509 *Table 1: Selection of physiologically important enzymes identified in the  $\Delta aceE$  and WT strains*  
510 *as showing significant regulation. Significant abundance changes of enzymes between strains*  
511 *are given as p-values obtained from ANOVA. The Regulation factor between the WT and  $\Delta aceE$*   
512 *(WT log2 area values subtracted from the  $\Delta aceE$  log2 area values) was determined from the z-*  
513 *normalized area values for WT and  $\Delta aceE$  samples. As threshold for significant regulation of*  
514 *proteins between strains a p-value below 0.05 was set. All significantly regulated proteins*  
515 *between WT and  $\Delta aceE$  can be found in suppl. Table 2.*

516

Gene ID	Description	P-value between strains	RF ( $\Delta aceE$ )
<b>Energy metabolism</b>			
Cg3048	PHOSPHATE ACETYLTRANSFERASE	3.05E-04	1.09
Cg3047	ACETATE KINASE	2.55E-03	0.87
<b>Carbohydrate metabolism</b>			
Cg0949	CITRATE SYNTHASE	4.12E-09	0.83
Cg0790	DIHYDROLIPOAMIDE DEHYDROGENASE	1.54E-02	0.59
Cg0791	PYRUVATE CARBOXYLASE	1.44E-05	0.64
Cg1737	ACONITASE	3.39E-03	0.33
Cg0766	ISOCITRATE DEHYDROGENASE	5.04E-05	0.40
Cg2421	DIHYDROLIPOAMIDE SUCCINYLTRANSFERASE	2.92E-02	0.25
Cg2840	SUCCINYL ACETATE COA-TRANSFERASE	2.06E-03	0.44
Cg0446	SUCCINATE DEHYDROGENASE A	4.47E-02	0.35
Cg1280	KETOGLUTARATE DEHYDROGENASE	2.70E-04	0.39
Cg1145	FUMARASE	8.32E-04	-0.39
Cg2613	MALATE DEHYDROGENASE	4.94E-04	-0.39
Cg1075	PHOSPHORIBOSYL PYROPHOSPHATE SYNTHASE	1.15E-02	0.36
Cg1780	PUTATIVE 6-PHOSPHOGLUCONOLACTONASE	5.45E-05	1.00
Cg2559	MALATE SYNTHASE	6.65E-04	0.53
Cg2192	MALATE:QUINONE OXIDOREDUCTASE	3.89E-02	0.24
Cg2521	LONG-CHAIN FATTY ACID COA LIGASE	4.74E-04	0.67
Cg0825	SHORT CHAIN DEHYDROGENASE; N-TERMINAL FRAGMENT	2.40E-04	1.01
Cg1373	GLYOXALASE	7.71E-04	-0.56
Cg0811	ACETYL/PROPIONYL COA CARBOXYLASE,	1.21E-04	0.35
Cg2560	ISOCITRATE LYASE	2.44E-07	1.13
Cg0802	BIOTIN CARBOXYLASE	3.02E-04	0.35
Cg1726	METHYLMALONYL-COA MUTASE	4.25E-02	0.33
Cg2091	POLYPHOSPHATE GLUCOKINASE	9.10E-03	0.63
Cg1268	GLYCOSYL TRANSFERASE	3.38E-02	0.34
Cg1381	1,4-ALPHA-GLUCAN BRANCHING ENZYME	1.63E-02	0.41
Cg2323	MALTOOLIGOSYL TREHALOSE SYNTHASE	2.50E-02	0.48
Cg1111	ENOLASE (2-PHOSPHOGLYCERATE DEHYDRATASE)	6.76E-04	-0.33
Cg1791	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE	2.17E-02	-0.15
<b>Nucleotide and amino acid metabolism</b>			
Cg0703	PUTATIVE GMP SYNTHASE	3.26E-02	0.28
Cg0700	IMP DEHYDROGENASE / GMP REDUCTASE	2.36E-02	0.57
Cg2964	INOSITOL-MONOPHOSPHATE DEHYDROGENASE	6.12E-03	0.54
Cg2953	BENZALDEHYDE DEHYDROGENASE	8.00E-07	1.39
Cg1581	GLUTAMATE N-ACETYLTRANSFERASE	3.43E-04	0.63
Cg0490	PYRROLINE-5-CARBOXYLATE REDUCTASE	4.82E-03	0.37
Cg1451	PHOSPHOGLYCERATE DEHYDROGENASE	5.29E-05	-0.41
Cg2586	GAMMA-GLUTAMYL PHOSPHATE REDUCTASE	1.91E-07	0.90
Cg1453	3-ISOPROPYLMALATE DEHYDROGENASE	3.91E-02	0.38
Cg1488	3-ISOPROPYLMALATE DEHYDRATASE	4.64E-02	0.22
Cg0303	2-ISOPROPYLMALATE SYNTHASE	1.03E-03	1.11
Cg1432	DIHYDROXY-ACID DEHYDRATASE	5.09E-04	0.35
Cg1436	ACETOHYDROXYACID SYNTHASE SMALL SUBUNIT	1.18E-03	0.48
Cg1435	ACETOLACTATE SYNTHASE	1.03E-03	0.58
Cg1806	S-ADENOSYLMETHIONINE SYNTHETASE	3.40E-02	0.76
Cg0860	ADENOSYLMETHIONINE SYNTHETASE	9.84E-03	0.77
Cg2833	O-ACETYL SERINE (THIOL)-LYASE	8.66E-05	0.26
Cg2437	THREONINE SYNTHASE	4.78E-03	0.40
Cg1338	HOMOSERINE KINASE	3.54E-03	0.57
Cg1337	HOMOSERINE DEHYDROGENASE	3.36E-04	0.60
Cg2779	PHOSPHOSERINE PHOSPHATASE	2.87E-02	0.34
Cg1713	DIHYDROOROTATE DEHYDROGENASE	1.53E-02	0.30
Cg2779	PHOSPHOSERINE PHOSPHATASE	2.87E-02	0.03
Cg1713	DIHYDROOROTATE DEHYDROGENASE	1.53E-02	0.06

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*Supplementary Table 1: Proteins identified as significantly regulated between mixed and separated samples of WT and  $\Delta aceE$  strain cells. Significant regulation of a protein between mixed and separated cells is given in the p-value and the Benjamini&Hochberg corrected adjusted p-value. The Regulation factor (RF) of a protein for the mixed samples (values of the*

524 *separated values are subtracted from the values of the mixed samples) was determined from*  
525 *the z-normalized log<sub>2</sub> area values of the separated and mixed samples from each strain. The*  
526 *threshold for significant regulation of proteins was set at a p-value of 0.05.*

527



Gene names	Description	P-value mix	adj. P-value mix	RF mix/separated (WT)	RF mix/separated ( $\Delta$ aceE)
Cg2994	PUTATIVE OR SECRETED MEMBRANE PROTEIN	1.79E-05	1.30E-02	NA	0.60
Cg3011	GROEL2 CHAPERONIN	1.07E-03	2.43E-01	-0.38	-0.21
Cg3306	50S RIBOSOMAL PROTEIN L9	1.47E-03	2.43E-01	-0.40	-0.21
Cg1560	EXCINUCLEASE ATPASE SUBUNIT	2.57E-03	2.43E-01	NA	0.51
Cg1841	PROBABLE ASPARTYL-TRNA SYNTHETASE	2.59E-03	2.43E-01	-0.87	-0.38
Cg3032	PUTATIVE SECRETED PROTEIN	2.89E-03	2.43E-01	-0.89	-0.31
Cg0414	CELL SURFACE POLYSACCHARIDE BIOSYNTHESIS	3.13E-03	2.43E-01	0.24	0.68
Cg0867	RIBOSOME-ASSOCIATED PROTEIN Y	3.14E-03	2.43E-01	0.24	0.68
Cg1476	THIAMINE BIOSYNTHESIS PROTEIN	3.16E-03	2.43E-01	0.77	0.18
Cg0842	PUTATIVE DNA HELICASE	3.86E-03	2.43E-01	0.52	0.55
Cg1206	PEP PHOSPHONMUTASE	3.97E-03	2.43E-01	-0.41	-0.46
Cg0239	HYPOTHETICAL PROTEIN	4.29E-03	2.43E-01	NA	-0.73
Cg1853	GLYCEROL-3-PHOSPHATE DEHYDROGENASE	4.46E-03	2.43E-01	NA	0.80
Cg1362	ATP SYNTHASE F0 SUBUNIT 6	5.03E-03	2.43E-01	0.38	1.05
Cg2835	Predicted acetyltransferase	6.36E-03	2.43E-01	-0.47	-0.23
Cg2120	SUGAR SPECIFIC PTS SYSTEM	6.46E-03	2.43E-01	-0.68	-0.33
Cg2705	MALTOSE-BINDING PROTEIN PRECURSOR	6.82E-03	2.43E-01	0.17	0.65
Cg2647	TRIGGER FACTOR	7.09E-03	2.43E-01	-0.43	-0.20
Cg1437	KETOL-ACID REDUCTOISOMERASE ILVC	7.68E-03	2.43E-01	-0.34	-0.19
Cg2137	GLUTAMATE SECRETED BINDING PROTEIN	8.54E-03	2.43E-01	0.93	NA
Cg1780	PUTATIVE 6-PHOSPHOGLUCONOLACTONASE	8.56E-03	2.43E-01	-0.47	-0.43
Cg1763	UNCHARACTERIZED IRON-REGULATED ABC-TYPE TRANSPOR	8.91E-03	2.43E-01	-0.63	-0.31
Cg0648	ADENYLATE KINASE	8.94E-03	2.43E-01	-0.28	-0.14
Cg2263	HYPOTHETICAL PROTEIN	9.00E-03	2.43E-01	NA	1.06
Cg1859	PUTATIVE SECRETED PROTEIN	9.93E-03	2.43E-01	-0.15	-0.44
Cg1333	ARGINYL-TRNA SYNTHETASE	1.05E-02	2.43E-01	-0.62	-0.22
Cg1538	DEPHOSPHO-COA KINASE	1.10E-02	2.43E-01	-0.59	-0.42
Cg2052	PUTATIVE SECRETED PROTEIN	1.13E-02	2.43E-01	-0.32	-0.94
Cg2026	HYPOTHETICAL PROTEIN	1.14E-02	2.43E-01	-1.09	-0.42
Cg2964	INOSITOL-MONOPHOSPHATE DEHYDROGENASE	1.17E-02	2.43E-01	-0.41	-0.39
Cg2911	ABC-TYPE MN/ZN TRANSPORT SYSTEM	1.19E-02	2.43E-01	0.19	1.67
Cg2873	PROLYL OLIGOPEPTIDASE	1.21E-02	2.43E-01	-0.97	-0.33
Cg0834	BACTERIAL EXTRACELLULAR SOLUTE-BINDING PROTEIN	1.23E-02	2.43E-01	0.27	0.49
Cg0007	DNA GYRASE SUBUNIT B	1.23E-02	2.43E-01	-0.49	-0.23
Cg0947	HYPOTHETICAL PROTEIN	1.25E-02	2.43E-01	-0.88	-0.38

Gene names	Description	P-value mix	adj. P-value mix	RF mix/separated (WT)	RF mix/separated ( $\Delta$ aceE)
<i>Cg3047</i>	ACETATE KINASE	1.27E-02	2.43E-01	-0.62	-0.63
<i>Cg1872</i>	HYPOTHETICAL PROTEIN	1.27E-02	2.43E-01	-0.34	-0.08
<i>Cg2521</i>	LONG-CHAIN FATTY ACID COA LIGASE	1.27E-02	2.43E-01	0.1	0.36
<i>Cg1588</i>	ARGININOSUCCINATE LYASE	1.35E-02	2.48E-01	-0.56	-0.21
<i>Cg0307</i>	ASPARTATE-SEMIALDEHYDE DEHYDROGENASE	1.40E-02	2.48E-01	-0.48	-0.42
<i>Cg2800</i>	PHOSPHOGLUCOMUTASE	1.41E-02	2.48E-01	-0.39	-0.2
<i>Cg1580</i>	N-ACETYL-GAMMA- GLUTAMYL-PHOSPHATE REDUCTASE	1.45E-02	2.48E-01	-0.48	-0.56
<i>Cg3132</i>	PUTATIVE MEMBRANE PROTEIN	1.59E-02	2.48E-01	-0.34	-0.33
<i>Cg1825</i>	TRANSLATION ELONGATION FACTOR P	1.60E-02	2.48E-01	-0.28	-0.2
<i>Cg2419</i>	LEUCINE AMINOPEPTIDASE	1.63E-02	2.48E-01	-1.28	-0.33
<i>Cg1586</i>	ARGININOSUCCINATE SYNTHASE	1.63E-02	2.48E-01	-0.33	-0.47
<i>Cg0691</i>	60 KDA CHAPERONIN	1.64E-02	2.48E-01	-0.2	-0.23
<i>Cg1487</i>	3-ISOPROPYLMALATE DEHYDRATASE LARGE SUBUNIT	1.67E-02	2.48E-01	-0.57	-0.53
<i>Cg2310</i>	GLYCOGEN DEBRANCHING ENZYME	1.68E-02	2.48E-01	NA	-0.77
<i>Cg0576</i>	DNA-DIRECTED RNA POLYMERASE BETA CHAIN	1.84E-02	2.66E-01	-0.32	-0.1
<i>Cg1463</i>	PUTATIVE GLUTAMYL-TRNA SYNTHETASE	1.94E-02	2.72E-01	-0.7	-0.23
<i>Cg2863</i>	PHOSPHORIBOSYLFORMYL GLYCINAMIDINE SYNTHASE	1.98E-02	2.72E-01	-0.51	-0.4
<i>Cg2102</i>	RNA POLYMERASE SIGMA FACTOR	1.99E-02	2.72E-01	NA	-0.35
<i>Cg1764</i>	UNCHARACTERIZED IRON-REGULATED ABC-TYPE TRANSPORTER	2.15E-02	2.87E-01	-0.64	-0.31
<i>Cg1404</i>	PROBABLE GLU-TRNA AMIDOTRANSFERASE	2.18E-02	2.87E-01	-0.42	-0.24
<i>Cg1531</i>	Zn-DEPENDENT HYDROLASE	2.39E-02	3.03E-01	-0.27	-0.13
<i>Cg2611</i>	MOLECULAR CHAPERONE, HSP 70 FAMILY	2.39E-02	3.03E-01	NA	0.85
<i>Cg1270</i>	PROBABLE O-METHYLTRANSFERASE	2.50E-02	3.04E-01	NA	-0.35
<i>Cg2954</i>	CARBONIC ANHYDRASE	2.52E-02	3.04E-01	0.22	0.29
<i>Cg2499</i>	GLYCYL-TRNA SYNTHETASE	2.61E-02	3.04E-01	-0.43	-0.16
<i>Cg2833</i>	O-ACETYL SERINE THIOL LYASE	2.64E-02	3.04E-01	-0.13	-0.14
<i>Cg1867</i>	PREPROTEIN TRANSLOCASE SUBUNIT SECD	2.65E-02	3.04E-01	NA	0.52
<i>Cg2661</i>	PUTATIVE DITHIOL-DISULFIDE ISOMERASE	2.73E-02	3.04E-01	0.21	0.22
<i>Cg3169</i>	PROBABLE PHOSPHOENOLPYRUVATE CARBOXYKINASE PROTEIN	2.73E-02	3.04E-01	-0.31	-0.17
<i>Cg2456</i>	ZN-RIBBON PROTEIN	2.76E-02	3.04E-01	-0.22	-0.35
<i>Cg0825</i>	SHORT CHAIN DEHYDROGENASE	2.77E-02	3.04E-01	-1.14	-0.09

Gene names	Description	P-value mix	adj. P-value mix	RF mix/separated (WT)	RF mix/separated ( $\Delta$ aceE)
<i>Cg0790</i>	DIHYDROLIPOAMIDE DEHYDROGENASE	2.88E-02	3.04E-01	-0.50	-0.34
<i>Cg3182</i>	TREHALOSE CORYNOMYCOLYL TRANSFERASE	2.89E-02	3.04E-01	0.10	0.76
<i>Cg2217</i>	RIBOSOME RECYCLING FACTOR	2.93E-02	3.04E-01	-0.28	-0.08
<i>Cg2117</i>	PHOSPHOENOLPYRUVATE PHOSPHOTRANSFERASE SYSTEM	2.98E-02	3.04E-01	-0.33	-0.19
<i>Cg2953</i>	BENZALDEHYDE DEHYDROGENASE	2.98E-02	3.04E-01	-0.67	-0.12
<i>Cg0387</i>	PUTATIVE ZINC-TYPE ALCOHOL DEHYDROGENASE	3.03E-02	3.04E-01	-0.39	-0.23
<i>Cg3050</i>	ACYLTRANSFERASE	3.16E-02	3.09E-01	-0.48	-0.45
<i>Cg0424</i>	PUTATIVE GLYCOSYLTRANSFERASE	3.17E-02	3.09E-01	NA	0.53
<i>Cg1574</i>	PHENYLALANYL-TRNA SYNTHETASE ALPHA CHAIN	3.21E-02	3.09E-01	-0.94	-0.11
<i>Cg2366</i>	CELL DIVISION GTPASE	3.29E-02	3.13E-01	-0.27	-0.31
<i>Cg0625</i>	SECRETED PROTEIN	3.32E-02	3.13E-01	-0.44	-0.20
<i>Cg2273</i>	RIBONUCLEASE III	3.54E-02	3.15E-01	NA	-0.34
<i>Cg2359</i>	ISOLEUCINE-TRNA LIGASE-LIKE PROTEIN	3.62E-02	3.15E-01	-0.90	-0.16
<i>Cg2963</i>	PROBABLE ATP-DEPENDENT PROTEASE	3.64E-02	3.15E-01	-0.33	-0.10
<i>Cg1880</i>	THREONYL-TRNA SYNTHETASE	3.64E-02	3.15E-01	-0.34	-0.25
<i>Cg2141</i>	DNA RECOMBINATION/REPAIR	3.64E-02	3.15E-01	-0.49	-0.16
<i>Cg1075</i>	PHOSPHORIBOSYL PYROPHOSPHATE SYNTHASE ISOZYME	3.69E-02	3.15E-01	-0.31	-0.21
<i>Cg2437</i>	THREONINE SYNTHASE	3.70E-02	3.15E-01	-0.39	-0.11
<i>Cg0438</i>	PUTATIVE GLYCOSYLTRANSFERASE	3.70E-02	3.15E-01	0.19	0.71
<i>Cg2363</i>	HYPOTHETICAL PROTEIN	3.79E-02	3.19E-01	-0.94	-0.29
<i>Cg2221</i>	TRANSLATION ELONGATION FACTOR TS	3.89E-02	3.24E-01	-0.24	-0.31
<i>Cg0193</i>	ENDOPEPTIDASE O	3.97E-02	3.24E-01	-0.50	-0.41
<i>Cg0766</i>	ISOCITRATE DEHYDROGENASE	3.98E-02	3.24E-01	-0.14	-0.17
<i>Cg0594</i>	50S RIBOSOMAL PROTEIN L3	4.15E-02	3.32E-01	-0.38	-0.19
<i>Cg1236</i>	THIOL PEROXIDASE	4.17E-02	3.32E-01	-0.57	-0.10
<i>Cg0807</i>	HYPOTHETICAL PROTEIN	4.33E-02	3.41E-01	-1.00	-0.28
<i>Cg3178</i>	POLYKETIDE SYNTHASE	4.61E-02	3.57E-01	-0.75	-0.02
<i>Cg3049</i>	PUTATIVE FERREDOXIN/FERREDOXIN-NADP REDUCTASE	4.64E-02	3.57E-01	-0.76	-0.43
<i>Cg0791</i>	PYRUVATE CARBOXYLASE	4.75E-02	3.60E-01	-0.22	-0.20
<i>Cg1365</i>	H <sup>+</sup> -ATPASE DELTA SUBUNIT	4.81E-02	3.60E-01	0.35	0.23
<i>Cg1228</i>	ABC-type cobalt transport system, ATPase component	4.99E-02	3.60E-01	-0.72	-0.03

531

532 *Supplementary Table 2: Proteins identified as significantly regulated between mixed and*  
533 *separated samples of WT and  $\Delta aceE$  strain cells. Significant regulation of a protein between*  
534 *WT and  $\Delta aceE$  strains cells is given in the p-value and the Benjamini&Hochberg corrected*  
535 *adjusted p-value. The Regulation factor (RF) of a protein (z-normalized log<sub>2</sub> WT values are*  
536 *subtracted from the z-normalized log<sub>2</sub>  $\Delta aceE$  values) was determined from the area values of*  
537 *each strain combining the separated and mixed samples. For each strain also the combined*  
538 *number of unique peptides from mixed and separated samples is given. The threshold for*  
539 *significant regulation of proteins was set at a p-value of 0.05.*

540

541

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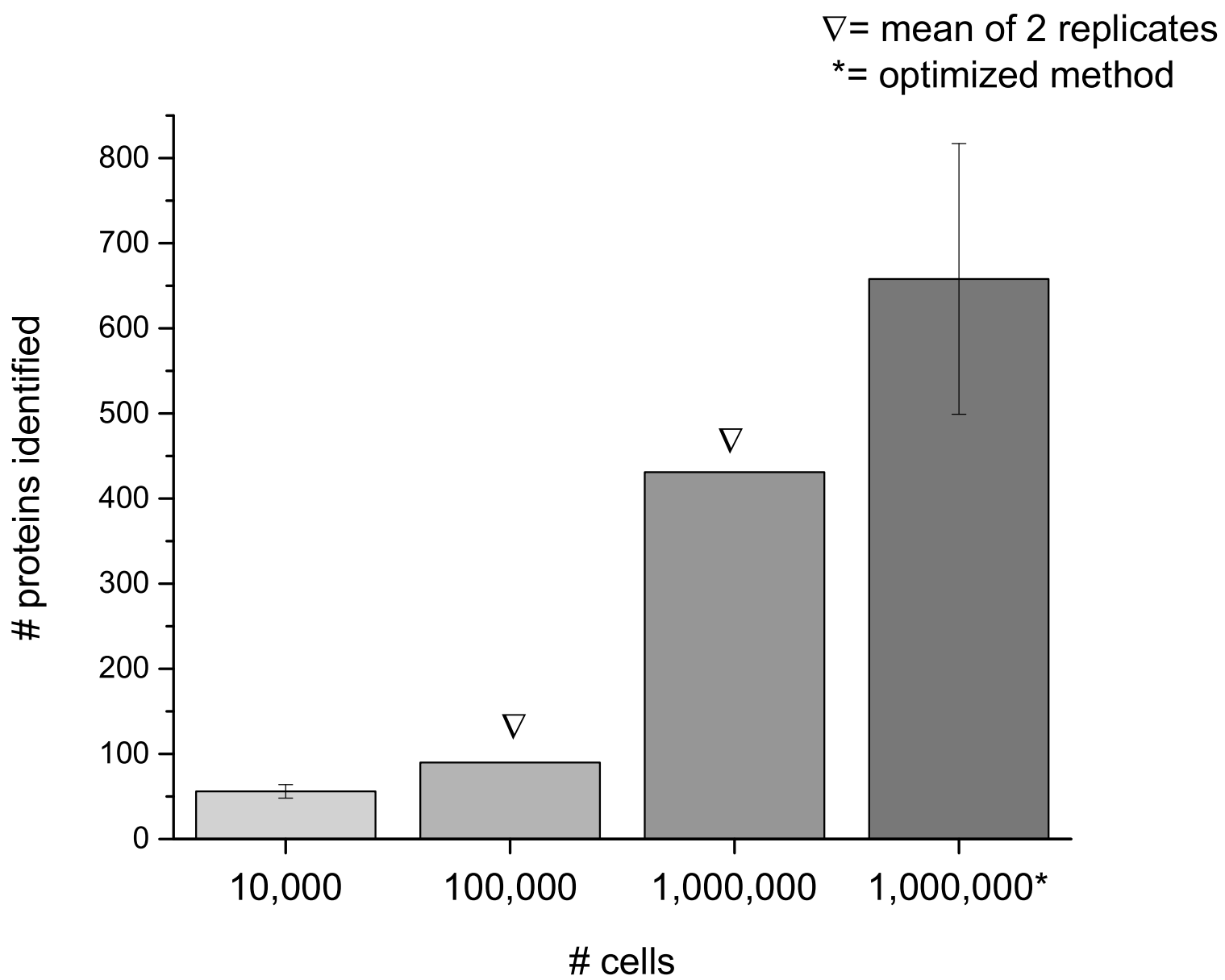
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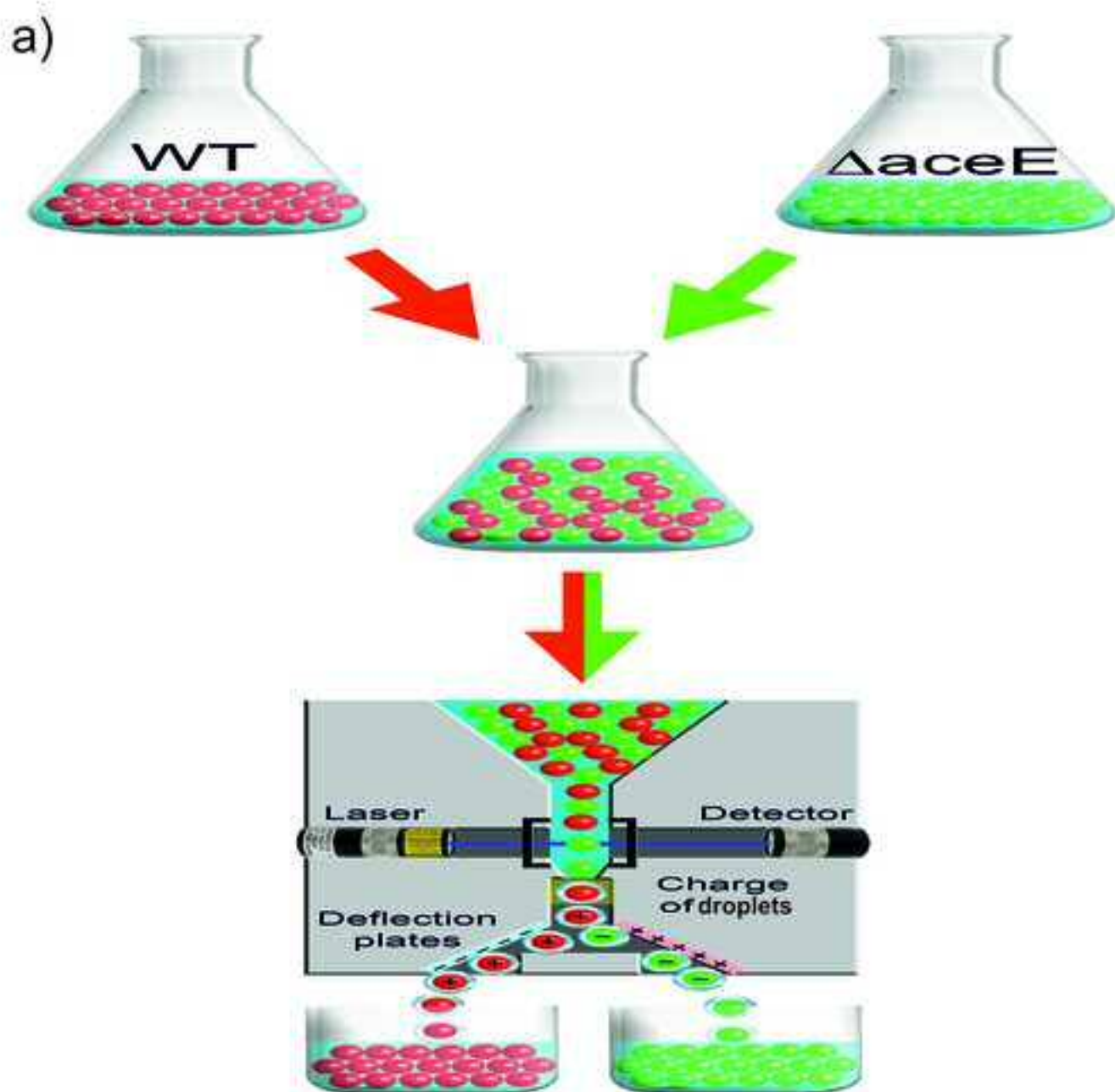
Gene ID	Description
<b>Energy metabolism</b>	
Cg3048	PHOSPHATE ACETYLTRANSFERASE
Cg3047	ACETATE KINASE
<b>Carbohydrate metabolism</b>	
Cg0949	CITRATE SYNTHASE
Cg0790	DIHYDROLIPOAMIDE DEHYDROGENASE
Cg0791	PYRUVATE CARBOXYLASE
Cg1737	ACONITASE
Cg0766	ISOCITRATE DEHYDROGENASE
Cg2421	DIHYDROLIPOAMIDE SUCCINYLTRANSFERASE
Cg2840	SUCCINYL ACETATE COA-TRANSFERASE
Cg0446	SUCCINATE DEHYDROGENASE A
Cg1280	KETOGLUTARATE DEHYDROGENASE
Cg1145	FUMARATE HYDRATASE
Cg2613	MALATE DEHYDROGENASE
Cg1075	PHOSPHORIBOSYL PYROPHOSPHATE SYNTHASE
Cg1780	PUTATIVE 6-PHOSPHOGLUCONOLACTONASE
Cg2559	MALATE SYNTHASE
Cg2192	MALATE:QUINONE OXIDOREDUCTASE
Cg2521	LONG-CHAIN FATTY ACID COA LIGASE
Cg0825	SHORT CHAIN DEHYDROGENASE; N-TERMINAL FRAGMENT
Cg1373	GLYOXALASE
Cg0811	ACETYL/PROPIONYL COA CARBOXYLASE,
Cg2560	ISOCITRATE LYASE
Cg0802	BIOTIN CARBOXYLASE
Cg1726	METHYLMALONYL-COA MUTASE
Cg2091	POLYPHOSPHATE GLUCOKINASE
Cg1268	GLYCOSYL TRANSFERASE
Cg1381	1,4-ALPHA-GLUCAN BRANCHING ENZYME
Cg2323	MALTOOLIGOSYL TREHALOSE SYNTHASE
Cg1111	ENOLASE (2-PHOSPHOGLYCERATE DEHYDRATASE)
Cg1069	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE
<b>Nucleotide and amino acid metabolism</b>	
Cg0703	PUTATIVE GMP SYNTHASE
Cg0700	IMP DEHYDROGENASE / GMP REDUCTASE
Cg2964	INOSITOL-MONOPHOSPHATE DEHYDROGENASE
Cg2953	BENZALDEHYDE DEHYDROGENASE
Cg1581	GLUTAMATE N-ACETYLTRANSFERASE
Cg0490	PYRROLINE-5-CARBOXYLATE REDUCTASE
Cg1451	PHOSPHOGLYCERATE DEHYDROGENASE
Cg2586	GAMMA-GLUTAMYL PHOSPHATE REDUCTASE
Cg1453	3-ISOPROPYLMALATE DEHYDROGENASE
Cg1488	3-ISOPROPYLMALATE DEHYDRATASE
Cg0303	2-ISOPROPYLMALATE SYNTHASE
Cg1432	DIHYDROXY-ACID DEHYDRATASE
Cg1436	ACETOHYDROXYACID SYNTHASE SMALL SUBUNIT
Cg1435	ACETOHYDROXYACID SYNTHASE
Cg1806	S-ADENOSYLMETHIONINE SYNTHETASE



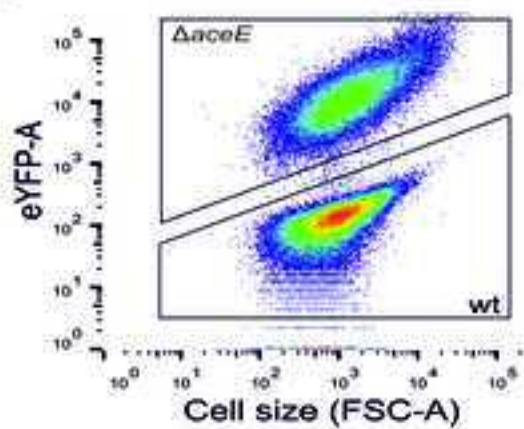


Figure

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b) Sorting of WT and  $\Delta aceE$  strain cells based on eYFP fluorescence



c) PCA of mixed and separated  $\Delta aceE$  and WT cells

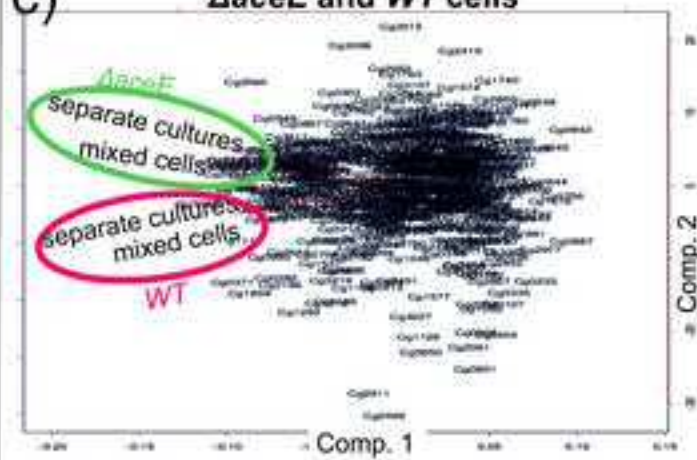
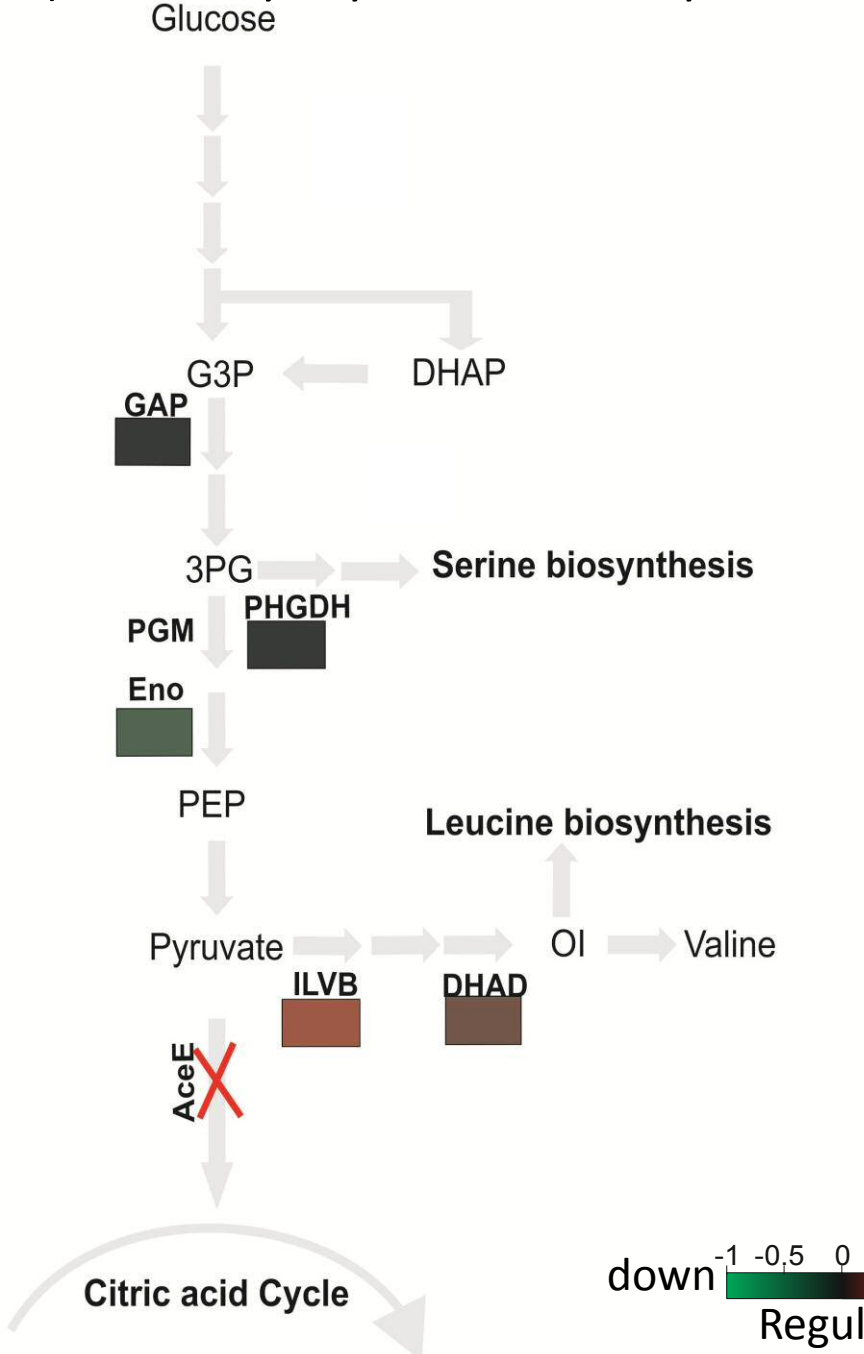


Figure 1  
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**a) Glycolysis and BCAA synthesis**



**b) Glyoxylate cycle and TCA**

