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1 Trends in systems biology for the analysis and engineering  
2 of *Clostridium acetobutylicum* metabolism

3

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17

18 **Key words:** *Clostridium acetobutylicum*, systems biology, proteomics,  
19 transcriptomics, fluxomics, metabolic engineering, biofuels

20

21 **Abstract:** *Clostridium acetobutylicum*, has received renewed interest worldwide as a  
22 promising producer of biofuels and bulk chemicals such as n-butanol, 1,3-propanediol,  
23 1,3-butanediol, isopropanol, and butyrate. To develop commercial processes for the  
24 production of bulk chemicals via a metabolic engineering approach, it is necessary to  
25 better characterize both the primary metabolism and metabolic regulation of *C.*  
26 *acetobutylicum*. Here, we review the history of the development of omics studies of *C.*  
27 *acetobutylicum*, summarize the recent application of quantitative/integrated omics  
28 approaches to the physiological analysis and metabolic engineering of this bacterium,  
29 and provide directions for future studies to address current challenges.

30

31

## 32 **Production of fuels and chemicals**

33 The production of fuels and bulk chemicals from renewable resources has attracted a  
34 great deal of attention in the last few decades as the atmospheric carbon dioxide level  
35 rises and petroleum resources become increasingly expensive. Bioethanol is currently  
36 commercialized as an alternative fuel in the market worldwide, but its characteristics  
37 do not meet the requirements of a perfectly ideal fuel as it has low energy content, low  
38 miscibility with diesel, limited blending rate with gasoline, high volatility and high  
39 corrosivity. Therefore, as alternatives to gasoline, advanced biofuels like C3-C5  
40 alcohols are believed to circumvent the current problems of ethanol. *Clostridium*  
41 *acetobutylicum* is a natural producer of industrially valuable chemicals like butanol,  
42 acetone and butyric acid and intensive efforts to engineer them for the efficient  
43 production of either kerosene precursors [1] or C2-C3-C4 alcohol mixtures as the only  
44 fermentation products have been undertaken [2-7]. Unfortunately, these bioprocesses  
45 are not economically viable for fuel applications due to high product toxicity for the  
46 producing bacteria, low yield and productivity and high cost of downstream processing  
47 [8]. However, a recombinant *C. acetobutylicum* strain was recently rationally  
48 engineered using a systems biology approach, and a continuous process was developed  
49 to produce n-butanol with high yield, titer and productivity [9], making production of  
50 this chemical at least economical to address the chemical market. Recombinant *C.*  
51 *acetobutylicum* strains have also been developed for the production of other chemicals  
52 of industrial interest, such as 1,3-propanediol [10], 2,3-butanediol [11], and butyrate  
53 [12]. To rationally engineer bacteria, different systems biology methods [13, 14] and  
54 synthetic biological tools [15] have been employed. We present here a historical review  
55 describing genome-scale metabolic models, transcriptomics, proteomics and  
56 metabolomics/fluxomics and their use for either the development of *C. acetobutylicum*  
57 strains for the production of bulk chemicals or for the complete physiological  
58 characterization of different mutants.

59

## 60 **Genome-scale metabolic reconstruction**

61 In batch culture, *C. acetobutylicum* exhibits biphasic behavior: during the acidogenic  
62 phase, acids such as acetate and butyrate are produced, decreasing the pH of the  
63 medium and forcing the organism to switch to the solventogenic phase, in which the  
64 acids are reassimilated, and solvents such as acetone, ethanol and butanol are produced

65 [16] (Figure 1 and Box 1). Furthermore, in phosphate-limited chemostat cultures, *C.*  
66 *acetobutylicum* can be maintained in three stable (i.e., without strain degeneration,  
67 contrary to nitrogen or  $Mg^{++}$  limitations) physiological states: i) an acidogenic state  
68 (production of acetic and butyric acids), ii) a solventogenic state (production of acetone,  
69 butanol, and ethanol) and iii) an alcohologenic state (production of butanol and ethanol  
70 but not acetone) (Figure 1 and Box 1) [17, 18].

71 A genome-scale metabolic reconstruction provides a highly mathematical, structured  
72 platform on which to understand the systems biology of metabolic pathways within a  
73 microorganism [19]. The integration of biochemical metabolic pathways with rapidly  
74 available, annotated genome sequences has resulted in the development of genome-  
75 scale metabolic models (GSMs). Simply put, these models correlate metabolic genes  
76 with metabolic pathways. In general, the more information about physiology,  
77 biochemistry and genetics that is available for the target organism, the better the  
78 predictive capacity of the reconstructed models. There exists a hierarchy of increasingly  
79 detailed *C. acetobutylicum* GSMs that have been progressively improved via the  
80 addition of new metabolic functions by making use of updated annotations [16, 17, 20-  
81 25]. Nonetheless, the latest GSM, *iCac967* [17], includes a higher number of  
82 experimentally validated reactions based on the biochemical characterization of several  
83 enzymes but fewer overall reactions than the previous GSM, *iCac802* [23], because the  
84 reactions with no strong literature- or experiment-based evidence, for example, aerobic  
85 reactions based on strict anaerobic characteristics of *C. acetobutylicum*, were removed  
86 [17]. The new or corrected reactions introduced in *iCac967* include those carried out  
87 by the bifurcating butyryl-CoA dehydrogenase; NADPH-dependent butanol  
88 dehydrogenases BdhA, BdhB and BdhC; NADH-dependent glutamate dehydrogenase;  
89 malate dehydrogenases; and ferredoxin-NADP<sup>+</sup> reductase.

90 The latest GSMs, namely, *iCac802* and *iCac967*, capture the biphasic behavior of the  
91 batch culture as a change in carbon uptake [17, 23]. However, the switch from  
92 acidogenesis to solventogenesis and the associated sporulation of the microorganism  
93 also affect the biomass (proteome and metabolome) composition [26], a phenomenon  
94 that has not been addressed by any of the current GSMs [13].

95 On the other hand, in phosphate-limited chemostat cultures performed at the same  
96 specific growth rate (i.e., a dilution rate of  $0.05\text{ h}^{-1}$ ), the biomass composition was  
97 shown to be relatively constant, regardless of the type of metabolism (acidogenic,

98 solventogenic or alcohologenic), which allowed increased accuracy of the flux  
99 distribution calculation [17] (Box 2).

100 While GSMs alone are quite useful for determining the metabolic potential of an  
101 organism, determination of the metabolic phenotype under various conditions requires  
102 the incorporation of additional information, such as transcriptomics or proteomics data.  
103 Transcriptomics, in particular, are the most comprehensive, and genomically complete  
104 sets of data that can be acquired. Incorporating transcriptomics data, the authors of  
105 *iCac802* established a method referred to as CoreReg [23]. CoreReg modified the flux  
106 bounds of reactions according to fold changes in gene expression, which were  
107 experimentally observed under butyrate and butanol stress conditions [27]. Subsequent  
108 interrogation of the model corroborated previous experimental findings [27, 28], i.e.,  
109 butanol stress strongly influences arginine metabolism, whereas butyrate stress imposes  
110 regulations on reactions in arginine and pyrimidine metabolism.

111

### 112 **Transcriptomics, proteomics and metabolomics**

113 Since the completion of the genome sequence of wild-type *C. acetobutylicum* ATCC  
114 824 in 2001 [29], genome-wide omics data have been obtained via various high-  
115 throughput methods (Figure 2) to better understand this organism based on non-targeted  
116 and non-biased analyses. In the early days, single omics methods were applied  
117 independently; however, accumulation of omics data and state-of-the-art techniques  
118 have led to the development of comprehensive multiomics approaches by the scientific  
119 community. Comparative multiomics studies have been conducted to analyze i)  
120 different metabolic states or transition states from acidogenesis to solventogenesis  
121 (time series in the case of batch fermentation); ii) metabolite stress, such as  
122 butanol/butyrate stress; iii) the utilization of different substrates; iv) key metabolic  
123 mutants versus wild-type strains; and v) others such as the development of omics  
124 methodologies (Table 1). It is worth taking account of the culture type in each study,  
125 as predominant studies were performed under batch conditions to simplify  
126 manageability. However, this type of culture condition can provide nebulous  
127 information, particularly in the case of solvent production and sporulation studies since  
128 these phenomena are coupled under batch conditions. In contrast, continuous cultures  
129 uncouple these processes while also keeping the specific growth rate constant (Box 2).

130

### 131 *Transcriptomics*

132 As described in detail for each study in Table 1, most transcriptomics analyses of *C.*  
133 *acetobutylicum* have been performed using DNA microarrays. From a historical point  
134 of view, the first transcriptomics research studies in *C. acetobutylicum* were published  
135 in 2003 and 2004 by a group led by E.T. Papoutsakis with seven pioneering studies  
136 based on microarray analyses [30-36]. In early days, only this research group and the  
137 collaborative group led by G. N. Bennett published transcriptomic studies that used  
138 microarrays [37, 38], and wide application of microarray technology in *C.*  
139 *acetobutylicum* research was only seen after 2010; in particular, the first two studies  
140 that used commercial monochromatic microarrays were published around this time,  
141 although the data were still presented as relative ratios in both studies [39, 40]. In 2015,  
142 our group pushed the limits of this technology by quantifying the absolute amounts of  
143 mRNA molecules per cell for all 3,916 ORFs, i.e., 3,738 ORFs on the chromosome and  
144 178 ORFs on the pSOL1 megaplasmid carrying key genes involved in solvent  
145 production [17, 41].

146 Although the early DNA microarray studies covered partial genomes and only  
147 furnished relatively restricted information, these large-scale analyses moved forward  
148 the understanding of the global transcriptional regulation of metabolic networks  
149 specifically related to sporulation [30, 42] and butanol stress [35, 36]. Furthermore,  
150 some of these transcriptomics data have been revisited by recent studies to correlate  
151 with their own data obtained by other omics approaches. For instance, a metabolomics  
152 study [26] revisited one of the initial transcriptomics studies of *C. acetobutylicum* [42]  
153 elucidating a similar subject, i.e., metabolic transition of *C. acetobutylicum* and the  
154 correlation of the expression of selected genes and changes in related metabolites; the  
155 data showed good agreement between tricarboxylic acid (TCA) cycle metabolites and  
156 related TCA cycle enzyme-encoding genes but disagreement for some amino acid  
157 pathways, which potentially suggests post-transcriptional level regulation.

158 Thus far, application of RNA sequencing (RNA-seq) for transcriptomic analysis of *C.*  
159 *acetobutylicum* has not been extensively reported; to the best of our knowledge, only  
160 five publications using RNA-seq data of *C. acetobutylicum* have appeared. The first  
161 report published in 2013 by E. T. Papoutsakis' research group investigated the  
162 mechanism by which 159 small noncoding RNAs (sRNAs) respond to metabolite stress  
163 [43]. In the past year, two research papers based on RNA-seq analysis were published  
164 to identify the transcriptional start sites (TSSs) of all genes expressed in *C.*

165 *acetobutylicum* [44] and to determine the transcriptional regulation of pentose sugar  
166 metabolism hierarchy in *C. acetobutylicum* [45].

167 As shown in the comparative analysis of microarray and RNA-seq data by  
168 Venkataramanan et al. [46], the transcriptomics analyses of *C. acetobutylicum* also  
169 potentially present variations originating only from the omics tools used. Therefore,  
170 this feature needs to be taken into account when comparing both previous and future  
171 data generated.

172

### 173 *Proteomics*

174 The first “systems-level” proteomics study on *C. acetobutylicum* could analyze only  
175 130 proteins by two-dimensional electrophoresis (2-DE) in 2002 to compare acidogenic  
176 and solventogenic chemostat cultures [47]. At that time, no other omics data for *C.*  
177 *acetobutylicum* were available. Sullivan and Bennett then performed 2-DE analysis  
178 [48] and compared their data to those of previous transcriptomics studies that were  
179 performed under the same culture conditions [30, 36]; for some genes, the protein and  
180 transcript levels were not correlated, implying that complex regulatory mechanisms  
181 might exist in *C. acetobutylicum*. For quantitative proteomics analysis of *C.*  
182 *acetobutylicum*, isobaric tags for relative and absolute protein quantification (iTRAQ)  
183 based data were incorporated into an integrative omics approach [46] but also revisited  
184 previous transcriptomics studies based on qualitative two-color microarray [27] and  
185 RNA-seq [43] analyses. All three of these omics data sets were obtained from batch  
186 cultures under butyrate/butanol stress, and a low correlation between mRNA and  
187 protein levels was revealed (Table 1).

188 For accurate high-throughput systems biology approaches, chemostat cultures present  
189 many advantages (see Box 2); thus, in many large-scale studies, chemostats were  
190 generally chosen as cultivation systems to provide reasonably comparable data [49].  
191 The first combined omics study on *C. acetobutylicum* under steady-state conditions was  
192 published in 2010 [50]. In this study, qualitative two-color microarray and 2-DE were  
193 carried out to analyze acidogenesis and solventogenesis; marked acidogenesis-specific  
194 expression of an operon comprising CA\_P0036-37, which was disregarded in previous  
195 batch-condition investigations, was discovered under steady-state conditions.

196 The first quantitative systems biology study was performed by growing *C.*  
197 *acetobutylicum* in chemostat cultures under acidogenic, solventogenic or alcohologenic  
198 conditions but always at the same specific growth rate [17]. Both the number of mRNA

199 molecules per cell (using quantitative one-color microarray) for all the genes and the  
200 number of protein molecules per cell (using quantitative label-free LC/LC-MS/MS  
201 high-definition mass spectrometry (HDMS)-based proteomics) for approximately 700  
202 ORFs were determined and associated with the results of fluxomic analysis. A large  
203 number of genes were observed to have less than 0.2 mRNA molecules per cell,  
204 indicating that there was either i) heterogeneity of expression among different cells or  
205 ii) a high mRNA degradation rate. Genes that showed <0.2 mRNA molecules per cell  
206 under all conditions were excluded from further analysis by the authors of the study. A  
207 linear relationship between the number of mRNA molecules per cell and the number of  
208 protein molecules per cell, regardless of metabolic state, was observed, allowing  
209 calculation of the absolute protein synthesis rates ( $s^{-1}$ ) for most of the 700 ORFs. These  
210 data were further used to characterize several metabolic mutants from quantitative  
211 transcriptomic and fluxomic data under the same physiological conditions [51-53].

212

### 213 *Metabolomics/Fluxomics*

214 The use of isotopic tracers (mostly  $^{13}\text{C}$ -labeling) has contributed to an improved  
215 understanding of *C. acetobutylicum* metabolism. In particular, bifurcation of the TCA  
216 cycle was demonstrated by two research groups, the group of M. R. Antoniewicz and  
217 the group of J. D. Rabinowitz, independently and almost simultaneously, by using  $^{13}\text{C}$ -  
218 labeled substrates [54, 55]. Subsequently, the former group published another  
219 metabolomics study by parallel labeling experiments using Uniformly labelled ( $\text{U-}^{13}\text{C}$ )  
220 and  $1\text{-}^{13}\text{C}$  substrates respectively that increase the number of redundant measurements  
221 for high resolution quantitative  $^{13}\text{C}$  metabolic flux analysis ( $^{13}\text{C}$ -MFA) in an  
222 experimentally valid (fitting all four labeling data sets) minimal model [56]. This study  
223 revealed new findings regarding the TCA cycle like the absence of notable fluxes  
224 between i)  $\alpha$ -ketoglutarate and succinyl-CoA/succinate and ii) fumarate/malate and  
225 oxaloacetate and the existence of an active pathway from pyruvate to fumarate via  
226 aspartate. In addition, the latter group revealed the redirection of carbon and electron  
227 (as alcohol production pathways particularly active in solventogenesis require  
228 consumption of NAD(P)H (Figure 1) [17]) fluxes due to changes in metabolic states  
229 from acidogenesis to solventogenesis (Box 1) [26] as well as hierarchies in sugar  
230 metabolism in *C. acetobutylicum* [57, 58]. These metabolomics/fluxomics-based  
231 findings enhanced the accuracy of recent GSMs of *C. acetobutylicum* [17, 23] regarding  
232 the TCA cycle and the pentose-phosphate pathway.



233

## 234 **Quantitative omics approaches in physiological studies and metabolic engineering**

235 Transcriptomics, proteomics, metabolomics and fluxomics have been separately used  
236 to elucidate important features of *C. acetobutylicum* metabolism as well as the  
237 interdependence of gene regulation (Figure 2). The integrative omics data have also  
238 provided comprehensive insights into *C. acetobutylicum* physiology in several ways  
239 including via characterization of acidogenic, solventogenic and alcohologenic  
240 metabolic pathways; the metabolic shift from acidogenesis to solventogenesis; the  
241 effects of butanol and butyrate stress; sporulation; and biofilm formation.

242 The recent studies that are highlighted in this section should provide up-to-date  
243 information regarding the application of quantitative/integrative omics approaches in  
244 physiological studies and in metabolic engineering of *C. acetobutylicum* (Figure 3).

245 A few studies have focused on the analysis of *C. acetobutylicum* physiology in steady-  
246 state chemostat cultures under acidogenic, solventogenic or alcohologenic conditions.  
247 By analyzing quantitative transcriptomic, proteomic, fluxomic and biochemical data,  
248 Yoo et al. elucidated the activity distributions of the five enzymes (AdhE1, AdhE2,  
249 BdhA, BdhB and BdhC) involved in the two-step butanol pathway (Figure 1) [17].  
250 Under solventogenic conditions, the first step was performed mainly by the NADH-  
251 dependent aldehyde dehydrogenase activity of AdhE1, whereas the second step was  
252 performed by the NADPH-dependent alcohol dehydrogenase activity of BdhB, as  
253 AdhE1 possesses very low alcohol dehydrogenase activity. On the other hand, under  
254 alcohologenic conditions, AdhE2 was responsible for NADH-dependent aldehyde and  
255 alcohol dehydrogenase activities. When *adhE1* was deleted, *adhE2* was expressed  
256 under solventogenic conditions, and AdhE2 could replace AdhE1 to catalyze the  
257 conversion of butyryl-CoA to butyraldehyde [51]. In contrast, when *adhE2* was deleted,  
258 AdhE1 could not replace AdhE2 under any of the metabolic conditions [51]. The roles  
259 of the two homologous *buk* (or *buk1*) and *buk2* genes were also elucidated using the  
260 same approach [52]. Buk has been reported to be the major butyrate kinase [52, 59].  
261 Additionally, deletion of *buk* could not be achieved without (partial) disruption of the  
262 *ptb* gene, most likely due to the toxicity of butyryl phosphate accumulation (Figure 1).  
263 Interestingly, AdhE2 was highly expressed in a  $\Delta buk\Delta ptb$  mutant under all steady-state  
264 metabolic conditions; a high butanol yield was obtained, and lactate was the main  
265 product in alcohologenesis [52]. Using a similar approach, CA\_P0037, a global  
266 regulator of central metabolism, was characterized by Nguyen et al. [53]. The metabolic

267 flux in acidogenesis and alcohologenesis changed significantly when CA\_P0037 was  
268 disrupted. Acids (lactate, acetate and butyrate) were the major products in  
269 alcohologenesis, whereas butanol and lactate levels increased in acidogenesis. These  
270 behaviors were consistent with the quantitative transcriptomic and proteomic profiles.  
271 There have been many studies on the behavior of *C. acetobutylicum* cells under butanol  
272 stress that have used transcriptomic data alone [28, 35, 60, 61] or in combination with  
273 proteomic [46] and fluxomic [23] data (Table 1). COntstraint-Based Reconstruction and  
274 Analysis (COBRA, metabolic modeling framework applying different constrains  
275 limiting achievable cellular function [62]) with additional constraints obtained from  
276 <sup>13</sup>C-MFA [63] was used to quantitatively analyze the effects of butanol stress on  
277 cellular metabolism i.e. low yield of biomass per ATP, slight effect on glycolysis but  
278 more on TCA cycle and serine/glycine pathway [64]. Via a metabolomics-based  
279 approach to investigate the intracellular biochemical changes in cells treated with  
280 butanol under acidogenic or solventogenic conditions, Wang et al. [65] demonstrated  
281 that i) a more active bifurcated TCA cycle, ii) an increased glycerol and amino acid  
282 formation, and iii) an altered fatty acid composition were key factors that contributed  
283 to the butanol tolerance of the cells. The same metabolomic approach was also  
284 employed to determine the key metabolites that might affect cell growth and butanol  
285 production [66]. Sugar metabolism and amino acid metabolism are favorable for cell  
286 growth and unfavorable for butanol production. Based on an analysis of dicarboxylate  
287 metabolism, ethylene glycol and citric acid supplementation during fermentation  
288 increased the formation of intermediates that promoted both cell growth and butanol  
289 production.

290 In addition to the physiological study of *C. acetobutylicum*, metabolomic comparison  
291 aided in the discovery of two new polyketides compounds, i.e. clostrienoic acid and  
292 clostrienose [67]. The authors also showed that, without these polyketides, the  
293 expression of genes involved in sporulation, carbohydrate transfer/transport and  
294 carbohydrate metabolism was downregulated, while that of genes involved in amino  
295 acid transport, sulfur metabolism, cofactor biosynthesis and stress response was  
296 upregulated.

297 In the field of metabolic engineering, the purpose is to construct strains that are capable  
298 of producing products of interest with the highest yield, productivity and selectivity. To  
299 support this work, mathematical models are usually used to predict, propose and/or  
300 characterize the metabolic pathways of all new mutants. For *C. acetobutylicum*, little

301 success has been achieved, mainly due to the lack of knowledge of the complex  
302 regulation system of the strain. Therefore, most mutants have been developed without  
303 mathematical and integrative system support. Here, we review some successful mutants  
304 that were constructed with the help of the omics integrative system. By analyzing  
305 metabolic fluxes, the group of S. Y. Lee reported that the direct pathway from acetyl-  
306 CoA via acetoacetyl-CoA and butyryl-CoA to butanol (Figure 1) could be improved by  
307 simultaneously disrupting the acetate and butyrate formation pathways and  
308 overexpressing *adhE1*<sup>D485G</sup> [68]. Consequently, a high yield (76% of the maximum  
309 theoretical yield) and high productivity (1.32 g/liter/h) of butanol were obtained under  
310 fed-batch conditions (Figure 3a).

311 Based on two studies from our group showing that i) *C. acetobutylicum* can use glycerol  
312 and divert most of the electron fluxes from hydrogen to alcohol formation in a glucose-  
313 limited chemostat culture [69] and ii) *Clostridium butyricum* uses a new pathway (a  
314 B12-independent glycerol dehydratase) to produce 1, 3 propanediol from glycerol [70],  
315 we engineered a *C. acetobutylicum* mutant continuously producing 1, 3 propanediol  
316 with a high yield, titer and productivity [71] (Figure 3b).

317 Collecting historical transcriptomic and qualitative proteomic analyses of *C.*  
318 *acetobutylicum* under high reducing power states (obtained by methyl-viologen  
319 supplementation or by chemostat culture at neutral pH on a mixture of glucose and  
320 glycerol or by inactivating *ptb* and *buk* [18, 52, 69, 72-74]), Liu et al. introduced an  
321 NADH-compensating module (comprising *alsD*, *bdhA* and *acr* genes) that triggers the  
322 mutant to produce 2,3-butanediol and simultaneously eliminates acetone production  
323 [7]. This mutant was subjected to proteomic analysis, and significant downregulation  
324 of the acetone pathway-encoding genes and high expression of *adhE2* (to replace  
325 AdhE1 and BdhB for solvent formation) were observed, which is very similar to results  
326 obtained under the other high reducing power states mentioned above (Figure 3c).

327 Regarding a more recent study, a *C. acetobutylicum* mutant was engineered to  
328 continuously produce *n*-butanol at very high yields with very high selectivity and  
329 productivity [9]. The straightforward engineering process was guided by i) fluxomics  
330 analysis using the GSM *iCac967* (which was developed by the same group) and the  
331 accurate calculation of all the electron fluxes [17] and ii) transcriptomics, proteomics  
332 and fluxomics analysis of a butyrate minus mutant [52]. This strain contained multiple  
333 deletions, including genes encoding for competing pathways such as butyrate formation  
334 ( $\Delta$ *ptb* $\Delta$ *buk*), lactate formation ( $\Delta$ *ldh*), acetone formation ( $\Delta$ *ctfAB*), and for a redox-

335 sensing transcriptional regulator (*ΔrexA*) that had been known to repress genes involved  
336 in the C4 formation pathway (*thlA*, *crt-bcd-etfAB-hbd* and *adhE2*). In addition to those  
337 deletions, gene substitutions *ΔthlA::atoB* and *Δhbd::hbd1* provided enzymes with  
338 improved characteristics that were effective at pulling the carbon flux toward the  
339 formation of butanol by making use of the high NADPH/NADP<sup>+</sup> ratio driving force.  
340 The strain obtained produced butanol at 83% of the maximum theoretical yield (Figure  
341 3d). The butanol-to-ethanol ratio increased 6-fold. However, the acetate formation  
342 pathway was still competing for carbon flux, as the strain produced acetate at low levels  
343 [9]. The intensive attempt to abolish all acid formation pathways, including acetate,  
344 was unsuccessful. Thus far, eliminating all by-products completely in *C.*  
345 *acetobutylicum* is not possible. Currently, the GSM *iCac967* is unable to explain this  
346 phenomenon. More studies must be performed to understand the physiology and  
347 regulatory systems prior to incorporating this knowledge into cell metabolic models for  
348 further strain engineering.

349

### 350 **Concluding Remarks and Future Perspectives**

351 The availability of improved GSMs in conjunction with quantitative transcriptomic and  
352 proteomic data now allows the acquisition of accurate fluxomic data when experiments  
353 are performed in chemostat cultures. These omics data allow (i) determination of the  
354 distribution of carbon and electron fluxes, (ii) elucidation of the different  
355 genes/enzymes involved in the primary metabolism of *C. acetobutylicum*, (iii)  
356 improved understanding of the regulation of *C. acetobutylicum* primary metabolism  
357 under different physiological conditions, (iv) the physiological characterization of  
358 several metabolic mutants and (v) the construction of a metabolically engineered strain  
359 that produces n-butanol at high yields.

360 With the availability of quantitative proteomic data (the numbers of protein molecules  
361 per cell) and of fluxes through each enzyme at the genome-scale level, it is now  
362 potentially possible to have access to the maximum *in vivo* turnover rate [75] for each  
363 metabolic enzyme of *C. acetobutylicum*. Such data will be very useful for developing  
364 an ME-Model [76] of *C. acetobutylicum* integrating metabolism with protein  
365 expression and proteome allocation constraints in order to predict the molecular  
366 composition of a cell or explain aspects of cell behavior that have remained elusive or  
367 require invocation of phenomenological relationships

368 Future work should also benefit from additional data on (i) the mechanism via which  
369 translation is regulated under different physiological conditions and (ii) the role of small  
370 RNAs in the regulation of central metabolism.

371

372

### 373 **Box 1. Physiological characteristics of *Clostridium acetobutylicum***

374 The growth of *C. acetobutylicum* in batch culture occurs in three different growth  
375 phases (Figure I).

376 Acids and most of the biomass are produced during the exponential growth phase. The  
377 accumulation of acids decreases the pH, which inhibits cell growth, causing the cells to  
378 enter the stationary phase. Solventogenesis is triggered during the transition to the  
379 stationary phase. Acids are then partially reassimilated, and acetone, butanol and  
380 ethanol (ABE solvents) are produced at a ratio of 3:6:1. At the end of the stationary  
381 phase, *C. acetobutylicum* sporulation and lysis occur. Biomass composition alteration  
382 during batch fermentation was not included in any of the current GSMs.

383

### 384 **Box 2. Advantages of chemostat cultures for systems biology studies**

385 *C. acetobutylicum* can be cultivated in continuous cultures (Figure I) under phosphate  
386 limitation and maintained in three stable metabolic states: **acidogenesis**, which involves  
387 the production of acids during growth at neutral pH on glucose; **solventogenesis**, which  
388 involves the production of ABE solvents during growth at low pH on glucose; and  
389 **alcohologenesis**, which involves the production of ethanol and butanol, but not  
390 acetone, during growth at neutral pH under conditions of high NAD(P)H availability.  
391 Steady-state continuous cultures, performed at the same specific growth rate (to avoid  
392 all the changes associated with changes in the specific growth rate observed in batch  
393 cultures), provide homogenous cells (Figure I) and relatively constant biomass  
394 compositions, which are key for obtaining accurate quantitative transcriptomic,  
395 proteomic, metabolomic and fluxomic data. Transcriptional start sites (TSSs), post-  
396 transcriptional processing sites (PSSs) and new regulators can be characterized, while  
397 the absolute protein synthesis rate, carbon and electron fluxes, and enzyme turnover  
398 rates can be measured at the whole-genome level.

399 **Figure 1. Central metabolism of *C. acetobutylicum*.** The green box indicates primary products  
400 under conditions of acidogenesis, whereas the red box indicates primary products under  
401 conditions of solventogenesis. The letters in red and italics indicate the corresponding genes.  
402 *ldh*, lactate dehydrogenase; *alsS*, acetolactate synthase; *alsD*, alpha-acetolactate decarboxylase;  
403 *hyd*, hydrogenase; *nfor*, ferredoxin:NAD(P)<sup>+</sup> oxidoreductase; *pfor*, pyruvate:ferredoxin  
404 oxidoreductase; *pdh*, pyruvate decarboxylase; *ack*, acetate kinase; *pta*, phosphotransacetylase;  
405 *adhE1*, aldehyde dehydrogenase; *adhE2*, bifunctional aldehyde/alcohol dehydrogenase; *bdh*,  
406 butanol dehydrogenase; *adc*, acetoacetate decarboxylase; *ctfAB*, CoA-transferase; *thl*, thiolase;  
407 *hbd*, 3-hydroxybutyryl-CoA dehydrogenase; *crt*, crotonase; *bcd*, butyryl-CoA dehydrogenase;  
408 *etf*, electron transfer flavoprotein; *buk*, butyrate kinase; *ptb*, phosphotransbutyrylase. Fd<sub>ox</sub>  
409 stands for oxidized ferredoxin, whereas Fd<sub>red</sub> stands for reduced ferredoxin.

410

411 **Figure 2. Integrative omics analyses for quantitative systems biology approaches.** Organic  
412 relationship between omics analyses performed on steady-state chemostat cultures.  
413 Representative studies' reference numbers of each level omics are shown in brackets.

414

415 **Figure 3: Omics based metabolic engineering of *C. acetobutylicum*, guided by the analysis**  
416 **of fluxomics (blue frame), transcriptomics (orange frame), proteomics (green frame) or**  
417 **combination data (mixed colors). The blue oval shape and the specified metabolism**  
418 **represent the mutant. The genotypes are highlighted in the purple rectangles. The**  
419 **increase or decrease of metabolites and mRNA transcripts (in green italic) is shown by up**  
420 **or down arrows, respectively. In figure 3b and 3d, carbon sources (glucose, glycerol) or**  
421 **additives fed to the cells were in orange rectangles.** BuOH, butanol; AcAc-CoA, acetoacetyl  
422 Coenzyme A; Acet-CoA, acetyl-CoA; But-CoA, Butyryl Coenzyme A; MV, methyl viologen;  
423 ACE, acetone; 2,3-BDO, 2,3 butanediol; EtOH, ethanol; BuOH/EtOH<sub>ratio</sub>, ratio of butanol to  
424 ethanol; BUT, butyrate; AC, acetone; LAC, lactate; 1,3-PDO, 1,3-propanediol; *pta*, gene  
425 encoding for phosphotransacetylase; *buk*, gene encoding butyrate kinase; *ctfAB*, genes encoding  
426 acetoacetyl-CoA:acyl-CoA transferase; *adc*, gene encoding acetoacetate decarboxylase;  
427 *adhE1*, gene encoding aldehyde dehydrogenase; *adhE2*, gene encoding bifunctional  
428 aldehyde/alcohol dehydrogenase; *bdhB*, gene encoding butanol dehydrogenase; *ptb*, gene  
429 encoding phosphotransbutyrylase; *ldh*, gene encoding lactate dehydrogenase; *rexA*: gene  
430 encoding redox sensing transcriptional regulator; *thlA*, gene encoding thiolase; *atoB*, synthetic  
431 gene encoding the thiolase from *Escherichia coli*; *hbd*, gene encoding an NADH dependent 3-  
432 hydroxybutyryl-CoA dehydrogenase; *hbd1*, gene encoding an NADPH dependent 3-

433 hydroxybutyryl-CoA dehydrogenase from *Clostridium kluyveri*; p<sub>ptb-alsD-RBSbdhA-RBSacr</sub>,  
434 plasmid expressing genes from the *ptb* promoter; p<sub>ptbadhE1<sup>D485G</sup></sub>, plasmid expressing a mutated  
435 (D485G) Adhe1; pSOL1: megaplasmid of *C. acetobutylicum* (a  $\Delta pSOL1$  strain is unable to  
436 produce solvents and spores); pSPD5(*dhaB1 dhaB2 dhaT*), plasmid expressing genes encoding  
437 a glycerol dehydratase (*dhaB1, dhaB2*) and 1,3-propanediol dehydrogenase (*dhaT*)

438

439 **Box 1, Figure I: Growth, product formation and cell differentiation of *C. acetobutylicum***  
440 **in batch culture.**

441

442 **Box 2, Figure I: Schematic representation of the experimental device to grow *C.***  
443 ***acetobutylicum* at a constant specific growth rate, under phosphate limitation, in different**  
444 **physiological states.**

445 **Table 1 Examples of omics studies of *C. acetobutylicum***

Category	Omics	Technique/Culture type/Strain	Main conclusion	Remarks	References
<b>i) Analysis of different metabolic states or transition states from acidogenesis to solventogenesis</b>					
	Proteomics	2-DE/Continuous/DSM 792 (mostly indistinguishable from wild-type ATCC 824)	Metabolic switch is accompanied by dramatic protein expression patterns such as solvent production, stress, serine pathway proteins	The first proteomics study of <i>C. acetobutylicum</i> using 2-DE covering 130 proteins for comparative analysis of acidogenesis and solventogenesis in chemostat cultures in 2002.	[47]
	Transcriptomics	DNA microarray/Batch/Wild-type (9 time points) and M5 (pSOL1 megaplasmid -lacking strain, 5 time points)	Metabolic shift from acidogenesis to solventogenesis, which was known to be strongly related to sporulation, accompanies striking global transcriptional changes, specifically in sporulation and solvent production genes	Qualitatively and quantitatively improved microarray analysis (covering 97% of whole genome) in 2005. The first proteomic study published in 2002 [47] was compared to verify consistency, such as for the serine pathway. The data were revisited to be correlated with an incipient metabolomics study in 2011 [26].	[42]
	Transcriptomics and proteomics	DNA microarray, 2-DE (21% coverage of cytoplasmic proteins, pI 4-7)/Continuous/Wild-type	Constant growth rate and exogenous parameters, except 2 different pH values to switch metabolic state, in chemostat enabled investigation of transcriptional and protein level changes of well-known genes as well as identification of striking	The first systems-level study of acidogenic (pH 5.7) and solventogenic (pH 4.5) chemostat cultures of <i>C. acetobutylicum</i> .	[50]



			expression changes in unknown proteins such as CA_P0036-37		
	Fluxomics	pH-dependent modeling/Continuous culture data and key regulatory information incorporated	From their model simulation for higher butanol yield, expression changes in multiple genes required (single gene is insufficient)	Kinetic model incorporating pH-dependent metabolic network and gene regulation, parameterized with steady-state analyses based on dynamic shift chemostat experiments, which are distinct from the pioneering stoichiometric model based on batch [77].	[78]
	Metabolomics	Liquid chromatography (LC)-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) & <sup>1</sup> H-nuclear magnetic resonance ( <sup>1</sup> H-NMR)/Batch/Wild-type	Globally redirected resources from biomass to solvent production in acid/solvent production pathways as well as core pathways, particularly the reductive TCA cycle, by the metabolic shift were mapped	Time-dependent intra/extracellular changes in metabolomes were described and compared with two revisited transcriptomic datasets from batch/chemostat cultures [42, 50].	[26]
	Transcriptomics	Microarray/Continuous/Wild-type	Antagonistic expression of <i>adhE1/2</i> and <i>thlA/B</i> , significantly induced stress response genes only during the shift, implies the trigger is decrease in the pH and not solvent production directly; contradictory to previous	Transcriptional analysis of acidogenic and solventogenic steady-state conditions as well as transient phase with 4 different pH points.	[79]

			stress study [42] under batch conditions		
	Transcriptomics	Microarray/Batch (methyl viologen added at exponential phase)/Wild-type	Old study performed with artificial electron carrier restricted to metabolite-level analysis [80] elucidated at the transcript-level and transcriptional regulation in solvent biosynthesis, i.e., overexpressed <i>adhE2</i> and repressed <i>sol</i> operon ( <i>adhE1</i> , <i>ctfAB</i> ), as well as <i>adc</i>	Transcriptional changes after addition of an artificial electron carrier (methyl viologen) were examined. Results that were partially similar to alcohologenesis were obtained, for instance, high <i>adhE2</i> in contrast to low <i>adhE1</i> expression.	[74]
	Transcriptomics	DNA Microarray/Batch/ <i>sigG</i> (Sporulation-Specific Sigma Factor G) mutant	$\sigma$ K is necessary in early sporulation prior to <i>spo0A</i> expression and is also necessary in late sporulation. New sporulation cascade model proposed	Study of the <i>sigG</i> mutant based on custom-built oligonucleotide Agilent Technologies 4x44k Microarray (the latest version in this field) for in-depth study of sporulation (and solvent formation) involving sigma factors	[81]
	Transcriptomics, proteomics and fluxomics	One-color DNA microarray & label-free comprehensive two-dimensional liquid chromatography -tandem mass spectrometry (LC/LC-MS/MS) high-definition mass spectrometry	Distribution of carbon/electron fluxes, different key role genes/enzymes and regulation in primary metabolism under different physiological conditions determined	Quantification of mRNA/protein molecules per cell for each ORF. Reconstruction of genome-scale model based on functional characterization of key enzymes/cofactor specificities. Three metabolic states, including alcohologenesis, were analyzed in chemostat cultures.	[17]

		(HDMS)/Continuous/Wild-type			
	Transcriptomics	DNA Microarray/Batch (4 time points)/ <i>C. acetobutylicum</i> B3 (CGMCC No. 5234) obtained after UV mutagenesis [82]	16.2% of the genome within biofilm cells was differentially expressed, in particular upregulated iron/sulfur uptake and glycolysis genes	One-color microarray assays of biofilm and planktonic cells of <i>C. acetobutylicum</i> , only relative fold changes presented.	[83]
<b>ii) Analysis of metabolite stress, such as butanol/butyrate stress</b>					
	Transcriptomics	DNA Microarray/Batch (2 different concentrations of butanol stress induction, 6 time points after stress induction)/groESL-overexpressing strain	Overexpression of solvent production genes triggered by butanol stress. Butanol concentration-dependent differential transcriptional expression of some genes reported	Early study to investigate transcriptional changes in response to butanol stress in a solvent-tolerant strain overexpressing the <i>groESL</i> operon (involved in regulation of heat shock gene expression).	[35]
	Transcriptomics	DNA Microarray/Batch (butanol stress-induced flask or bioreactor)/ <i>spo0A</i> (the regulator of endospore formation) -overexpressing vs knockout strains	Overexpression of <i>spo0A</i> resulted in increased butanol tolerance accompanied by upregulated genes involved in butyryl-CoA/butyrate synthesis/assimilation but did not dramatically change solvent production despite detection of the	Early study to investigate transcriptional changes in response to butanol stress in a solvent-tolerant strain overexpressing <i>spo0A</i> and comparison to <i>spo0A</i> knockout strain. Some contradictory results between pH-controlled batch culture in bioreactor and butanol-challenged batch culture in flask likely due to uncontrolled cell differentiation.	[36]

			transcriptional alteration of related genes		
	Transcriptomics	RNA-seq/Batch (3 different concentrations of butanol or butyrate stress induction, 4 time points after stress induction)/WT	Use of RNA-seq enabled identification of 7.5% of the RNA-seq reads mapped to non-annotated ORFs, which are responses to metabolite stress. Comprehensive understanding of their roles in regulatory network is required	The first RNA-seq-based genome-scale analysis, where 159 sRNAs, 113 sRNAs previously computationally predicted [84] plus 46 sRNAs newly experimentally identified from RNA-seq data, were examined.	[43]
	Transcriptomics and proteomics	Isobaric tag for relative and absolute quantitation (iTRAQ)/Batch (3 different concentrations of butanol or butyrate stress induction, 3 time points after stress induction)/WT	Post-transcriptional regulation triggered by metabolites stress in <i>C. acetobutylicum</i>	Quantitative analysis of the proteome, covering 15% of the genome, was compared to previously published microarray [27] and RNA-seq [43] data taken from the same master cultures.  Poor correlation was shown (31%), ultimately suggesting post-transcriptional regulation under conditions of metabolite stress.	[46]
<b>iii) Utilization of different substrates</b>					
	Transcriptomics	DNA Microarray/Batch (tested carbon sources:	Sugar-specific transcriptional level regulation of the transport system and utilization metabolism	The first one-color microarray (3,842 ORFs) study; however, only heatmaps were presented. Comparative transcriptomic analysis of cells	[40]

		glucose, mannose, galactose, fructose, arabinose, xylose, sucrose, lactose, maltose, cellobiose or starch)/WT		growing on 11 different carbohydrates. Specifically, pentose metabolism-related gene annotations were updated based on genome-wide data.	
	Transcriptomics	DNA microarray/Batch on mixture of glucose and xylose (4 time points)/WT	Putative xylose degradation mechanism involving two operons proposed, catabolite repression elements (CRE) transcriptionally inhibited by binding of catabolite control protein CcpA to specific site identified	Transcriptional analysis of diauxic growth on mixture of glucose and xylose.	[85]
	Proteomics	Two-dimensional - liquid chromatography - tandem mass spectrometry (2D-LC-MS/MS)/Batch (glucose vs xylose)/WT	22 significantly differentially expressed proteins, whereas 649 proteins were commonly expressed. Proteins related to cellular motility as well as AdhE2 were upregulated on glucose relative to xylose. Highest number (894) of proteins identified in this organism to date of this work	The first non-2DE based proteomic (MS-based shotgun) study, which identified 894 different proteins. This proteomic approach was used to compare cultures using glucose or xylose as the sole carbon source. Overexpression of chemotaxis proteins was observed in agreement with transcriptional data in [85]; however, upregulation of AdhE2, the gene product of CA_P0035 (note: in this paper wrongly annotated as AdhE1), HBD in	[86]

				primary metabolism seen only in this proteomics study.	
	Proteomics	2D-LC-MS/MS/Batch (xylose)/WT	17 significantly differentially expressed proteins between exponential and stationary phases	MS-based shotgun study comparing cultures grown on xylose in exponential and stationary phases. Interestingly, upregulated ThIA in stationary phase was reported, while gene products of <i>hbd</i> , <i>crt</i> and <i>bcd</i> were not reported as significantly changed; unfortunately metabolite profiles were not included for further analysis.	[87]
	Transcriptomics	DNA microarray/Batch (glucose + xylose)/ <i>ccpA</i> -inactivated strain vs WT	<i>CcpA</i> plays a role in carbon catabolite repression, inactivation of <i>ccpA</i> influenced solvent production genes (upregulation of <i>sol</i> operon) as well as sporulation genes ( <i>ccpA</i> is necessary for efficient sporulation)	Transcriptionally affected genes, in particular in carbohydrate metabolism, by inactivation of <i>ccpA</i> encoding catabolite control protein.	[88]
	Fluxomics and metabolomics	Gas chromatography mass spectrometry (GC-MS)/ <i>xfp</i> (xylulose-5-phosphate/fructose-6-phosphate phosphoketolase) inactivated/overexpressed strains	Increased xylose concentration led to increased contribution of phosphoketolase pathway relative to that of pentose-phosphate pathway. <i>xfp</i> -inactivated mutant still had phosphoketolase activity; on the	Use of <sup>13</sup> C metabolic flux analysis to characterize xylose metabolism. Quantification of xylose catabolic pathways fluxes.	[89]

			other hand, overexpression resulted in higher acetate production		
	Metabolomics and fluxomics	One-dimensional proton nuclear magnetic resonance spectrum ( <sup>1</sup> H NMR) and liquid chromatography mass spectrometry (LC-MS)/Batch (glucose, glucose + xylose, glucose + arabinose, and xylose + arabinose)/WT	Hierarchy in <i>C. acetobutylicum</i> pentose metabolism: Arabinose is preferred to xylose, and more flux through the phosphoketolase pathway than through the pentose-phosphate pathway	Metabolomics and quantitative metabolic flux analysis (MFA) to determine the hierarchy in pentose sugar metabolism. Use of the phosphoketolase pathway in pentose metabolism was confirmed by metabolomic and fluxomic analyses.	[57]
	Metabolomics	Liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS)/Batch (xylose with high concentration of sodium)/WT	Accumulation of ATP and NADH, inhibition of PPP and glycolytic pathways observed in high sodium culture; however, detection of constant NADP <sup>+</sup> -to-NADPH ratio detected may be related to solvent-specific productivity, which was not affected	A recent quantitative metabolomics characterization of <i>C. acetobutylicum</i> grown on xylose with a high concentration of sodium to understand high sodium-mediated inhibition of cell proliferation but similar solvent productivity.	[90]
<b>iv) Key metabolic mutants versus the wild-type strain</b>					
	Transcriptomics	DNA microarray (1,019 genes including all 178 pSOL1 genes)/Batch/ <i>spo0A</i> -	The majority of gene expression patterns in SKO1 are similar to those in <i>B. subtilis</i>	The first omics study of <i>C. acetobutylicum</i> using microarray technology to correlate gene expression patterns.	[30]

		inactivated SKO1 strain and megaplasmid pSOL1 loss M5 strain (both asporogenous and nonsolventogenic)	Downregulation of all solvent formation genes and <i>sigF</i> (sporulation-specific sigma factors)	and specific phenotypes at a large-scale and to determine transcriptomic similarities/ differences between the 2 strains as well as those between <i>C. acetobutylicum</i> and <i>B. subtilis</i> (cf. Until this study, transcript-level understanding relied only on studies in <i>Bacillus subtilis</i> , a distantly close but physiologically quite dissimilar organism).	
	Proteomics	2-DE/Batch (4 time points)/SKO1	23 proteins were identified, 5 (e.g., GroEL, Bcd) proteins were detected in more than one location to indicate post-translational modifications of those proteins Protein profile of Adc confirmed regulation of this protein by Spo0A	Determine how <i>C. acetobutylicum</i> proteome changed over time (acidogenesis, transition, and solventogenesis) and how inactivation of Spo0A impacts global protein expression pattern. Revisited previously published microarray data [30, 36] for comparison.	[48]
	Transcriptomics	DNA microarray/Batch (exposure to oxygen)/ $\Delta perR$	Identification of some PerR regulons. Upregulation of repair and biogenesis of DNA, Fe-S cluster genes, downregulation of butyrate formation gene due to exposure to oxygen	Transcriptional responses of 3,840 ORFs (99.8% coverage of all ORFs ) of <i>perR</i> (peroxide response regulator) mutant under sublethal levels of oxygen exposure condition were analyzed by microarray	[91]
	Proteomics	2-DE and peptide detection	Differentially expressed proteins between the 2 strains are primarily	Proteome reference map with	[95]



		by MS/MS/Batch/Type strain DSM 1731 and Rh8 (butanol-tolerant mutant)	related to protein folding, solvent production, amino acid/nucleotide metabolism, protein synthesis or transport	564 different proteins was established, and this map was used in subsequent studies [92-94].	
	Genomics and transcriptomics	DNA microarray/Batch/EA 2018 (CCTCC M 94061, non-sporulating and higher butanol production ability of EA2018)	2215 genes were differentially expressed at the transcriptional level. Genome-level changes in the expression of <i>adhE2</i> , hydrogenase gene ( <i>hydA</i> ), and <i>spo0A</i> , which are key genes in solvent production and sporulation	The first use of commercial monochromatic microarrays (Agilent), associated with the complete genome sequence of the EA 2018 hyper-butanol-producing strain.	[39]
	Transcriptomics and proteomics	DNA microarray and 2-DE/Batch/ <i>fur</i> -inactivated strain	High production of riboflavin with overexpression of riboflavin biosynthesis operon. Iron-transport systems are regulated by Fur under presence of iron. Iron limitation and inactivation of <i>fur</i> altered expression of <i>ldhA</i> and the main flavodoxin-encoding gene	Wild-type and <i>fur</i> (ferric uptake regulator) mutant grown under iron-repleted and depleted conditions were analyzed by transcriptomic and proteomic approaches.	[96]
	Transcriptomics	DNA Microarray/Continuous/ $\Delta ptb$ , $\Delta pta$ , $\Delta adc$ , $\Delta adc\Delta pta$ strains	<i>ptb</i> mutant cultivated in chemostat in this study exhibited a different product pattern (similar to [52],	Several metabolic (in acid and acetone pathways) mutants grown in chemostat cultures were analyzed for transcriptional	[98]

			<p>butanol as the major product in acidogenesis and solventogenesis) than that from the batch culture (ethanol as the major product) [97]. Strikingly upregulated <i>adhE2</i> detected in <math>\Delta pta</math> and <math>\Delta adc\Delta pta</math> under both metabolic conditions. However, in <math>\Delta ptb</math>, only slightly increased expression of this gene in acidogenesis was claimed, which does not agree with [52], which showed high expression of the gene</p>	<p>changes under acidogenesis and solventogenesis</p>	
	<p>Transcriptomics, proteomics and fluxomics</p>	<p>DNA microarray/Continuous culture/<math>\Delta adhE1</math>, <math>\Delta adhE2</math> strains</p>	<p>For butanol formation, the key enzyme is AdhE1 under solventogenesis and AdhE2 under acidogenesis and alcohologenesis. AdhE1 can be partially replaced by upregulated AdhE2 under solventogenesis, whereas AdhE2 is essential under alcohologenesis</p>	<p>Elucidation of the roles of <i>adhE1</i> and <i>adhE2</i> in the primary metabolism of <i>C. acetobutylicum</i> using a quantitative systems biology approach.</p>	<p>[51]</p>

	Transcriptomics, proteomics and fluxomics	DNA microarray/Continuous/CA_P0037 inactivated strain	Changed flux profiles in acidogenesis (lactate, butyrate, and butanol) with overexpressed main flavodoxin gene and in alcohologenesis (butyrate, acetate, and lactate) with upregulated <i>ldhA</i> and downregulated <i>adhE2</i> . Self-regulated expression mechanism for CA_P0037 based on high expression of CA_P0037-CA_P0036 operon in the mutant.	Identification and characterization of CA_P0037, a novel global regulator of <i>C. acetobutylicum</i> metabolism.	[53]
	Transcriptomics, proteomics and fluxomics	DNA microarray/Continuous/ $\Delta buk\Delta ptb$	In acidogenesis, butanol is mainly produced along with a new metabolite, 2-hydroxy-valerate. In solventogenesis, a high yield of butanol formation observed. In alcohologenesis, lactate was the major product. Overexpression of <i>adhE2</i> detected in all metabolic conditions	Analysis of the metabolic flexibility of a butyrate pathway mutant of <i>C. acetobutylicum</i> using a quantitative systems biology approach.	[52]

	Transcriptomics and metabolomics	Liquid chromatography-high-resolution mass spectroscopy (LC-HRMS) and RNA-seq/Batch/ $\Delta pks$	Impacts of polyketide production in <i>C. acetobutylicum</i> : Slightly increased solvent production but no significant transcriptional change in the <i>sol</i> operon in $\Delta pks$ . The polyketides act like triggers of sporulation related to sigma K expression	In $\Delta pks$ (deletion mutant of the gene encoding polyketide synthase), 1) loss of polyketides was observed, and the polyketides were identified (clostrienoic acid and clostrienose) using a metabolomics approach; and 2) reduced sporulation was observed, and the important role of sigma K was suggested based on RNA-seq transcriptomic data.	[67]
	Genomics and fluxomics	In silico metabolic flux analysis with iCac802 [23]/Batch/hyper-ABE-producing BKM19 strain	Mutation of the thiolase in this strain led to higher activity than WT. Accordingly, an enhanced flux in butanol production was obtained	Genomics of the hyper-ABE-producing BKM19 strain and <i>in silico</i> metabolic flux analysis of this strain using genome-scale model iCac802.	[99]
<b>v) Others</b>					
	Transcriptomics	DNA microarray/Batch/WT and M5 (pSOL1 megaplasmid lost)	Framework for designing and validating DNA microarrays using a commercial process (Agilent) developed in 2007 and the resulting 22K microarrays was applied to the M5 strain for experimental validation	DNA microarray development strategy and experimental validation to cover all 3,916 ORFs (tRNA and rRNA excluded) of <i>C. acetobutylicum</i>	[100]
	Proteomics	2-DE/Continuous/WT	Respective SOPs for culture supernatant and cell pellet for 2-DE of <i>C. acetobutylicum</i> are drawn as	With this 2-DE-based standard operating procedure (SOP), 736 intracellular and 324 extracellular protein spots were observed, and	[101]

			shown in Figure 10 in the original paper	the SOP was applied in a subsequent study [50].	
	Metabolomics and fluxomics	<sup>13</sup> C-based isotopomer analysis/Batch/WT	Although genome annotation of <i>C. acetobutylicum</i> lacked most corresponding TCA cycle genes, this study demonstrated a complete bifurcated TCA cycle. In detail, oxaloacetate flows to succinate both through citrate (clockwise)/ $\alpha$ -ketoglutarate and via malate/fumarate (counterclockwise)	The complete bifurcated TCA cycle was mapped, and fluxes in central metabolism were quantitatively determined using isotopic tracer data. Very similar approach and results to [54] and published slightly earlier.	[55]
	Metabolomics and fluxomics	<sup>13</sup> C-based isotopomer analysis/Batch/WT	Split TCA cycle where only Re-citrate synthase contributes to $\alpha$ -ketoglutarate production; the oxidative pentose-phosphate pathway (oxPPP) is inactive	Determination of central metabolism pathway, particularly the TCA cycle, in <i>C. acetobutylicum</i> using isotopic tracer data, simultaneously published with [55].	[54]
	Proteomics	iTRAQ tags with RP–RP LC-MS/Batch under butanol stress/WT	One of the recently developed proteomic shotgun analysis methods for quantifying changes in peptide/protein expression level was applied to <i>C. acetobutylicum</i> . The addition of calcium carbonate enhanced cell lysis and ultimately	This workflow using iTRAQ tags with reversed-phase/reversed-phase liquid chromatography mass spectrometry (RP–RP LC-MS) was used to identify and quantify 566 unique proteins and was used in subsequent studies [46].	[102]

			increased protein recovery with this workflow.		
	Metabolomics and fluxomics	<sup>13</sup> C-based isotopomer analysis/ Batch/WT	In this study, no measurable flux between $\alpha$ -ketoglutarate and succinyl-CoA, succinate and fumarate, and malate and oxaloacetate was detected, which is contradictory to [55], and it was claimed that succinate must be mostly reused for succinyl-CoA by CoA-transferase based on the low succinate secretion rate in [55]	A minimal metabolic network model for fluxomic analysis was established and quantitatively validated by parallel labeling experiments.	[56]
	Metabolomics	Gas chromatography/time-of-flight mass spectrometry/ Batch/WT	Use of pure methanol was better than the frequently used solvent mixture acetonitrile/methanol/water (2:2:1, v/v/v) at -20 °C; extraction solvents changed metabolomics profiles; the method developed in this study could be applied in atmospheric conditions without significant changes in the metabolome of <i>C. acetobutylicum</i>	Metabolome methodology development for atmospheric metabolite sampling and processing of the strict anaerobe <i>C. acetobutylicum</i> .	[103]

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452

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