Trends in Systems Biology for the Analysis and Engineering of Clostridium acetobutylicum Metabolism

Minyeong Yoo, Ngoc-Phuong-Thao Nguyen, Philippe Soucaille

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

Minyeong Yoo[1], Ngoc-Phuong-Thao Nguyen[2], & Philippe Soucaille[1,3,4,5,6 *]

Affiliations:

[1] BBSRC/EPSRC Synthetic Biology Research Centre, School of Life Sciences, Centre for Biomolecular Sciences, University of Nottingham, United Kingdom.

[2]Tan Tao University, School of Medicine, Tan Duc e-City, Duc Hoa, Long An, Vietnam.

[3]Université de Toulouse, INSA, UPS, INP, TBI, Toulouse, France.

[4]INRA, UMR792, Toulouse, France.

[5]CNRS, UMR5504, Toulouse, France.

[6]Metabolic Explorer, Biopôle Clermont-Limagne, Saint Beauzire, France.

Correspondence: soucaille@insa-toulouse.fr (P. Soucaille)

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

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

Production of fuels and chemicals

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

Genome-scale metabolic reconstruction

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

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

A genome-scale metabolic reconstruction provides a highly mathematical, structured platform on which to understand the systems biology of metabolic pathways within a microorganism [19]. The integration of biochemical metabolic pathways with rapidly available, annotated genome sequences has resulted in the development of genome-scale metabolic models (GSMs). Simply put, these models correlate metabolic genes with metabolic pathways. In general, the more information about physiology, biochemistry and genetics that is available for the target organism, the better the predictive capacity of the reconstructed models. There exists a hierarchy of increasingly detailed *C. acetobutylicum* GSMs that have been progressively improved via the addition of new metabolic functions by making use of updated annotations [16, 17, 20-25]. Nonetheless, the latest GSM, *i*Cac967 [17], includes a higher number of experimentally validated reactions based on the biochemical characterization of several enzymes but fewer overall reactions than the previous GSM, *i*Cac802 [23], because the reactions with no strong literature- or experiment-based evidence, for example, aerobic reactions based on strict anaerobic characteristics of *C. acetobutylicum*, were removed [17]. The new or corrected reactions introduced in *i*Cac967 include those carried out by the bifurcating butyryl-CoA dehydrogenase; NADPH-dependent butanol dehydrogenases BdhA, BdhB and BdhC; NADH-dependent glutamate dehydrogenase; malate dehydrogenases; and ferredoxin-$NADP^+$ reductase.

The latest GSMs, namely, *i*Cac802 and *i*Cac967, capture the biphasic behavior of the batch culture as a change in carbon uptake [17, 23]. However, the switch from acidogenesis to solventogenesis and the associated sporulation of the microorganism also affect the biomass (proteome and metabolome) composition [26], a phenomenon that has not been addressed by any of the current GSMs [13].

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

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

While GSMs alone are quite useful for determining the metabolic potential of an organism, determination of the metabolic phenotype under various conditions requires the incorporation of additional information, such as transcriptomics or proteomics data. Transcriptomics, in particular, are the most comprehensive, and genomically complete sets of data that can be acquired. Incorporating transcriptomics data, the authors of *i*Cac802 established a method referred to as CoreReg [23]. CoreReg modified the flux bounds of reactions according to fold changes in gene expression, which were experimentally observed under butyrate and butanol stress conditions [27]. Subsequent interrogation of the model corroborated previous experimental findings [27, 28], i.e., butanol stress strongly influences arginine metabolism, whereas butyrate stress imposes regulations on reactions in arginine and pyrimidine metabolism.

Transcriptomics, proteomics and metabolomics

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

Transcriptomics

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

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

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

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

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

Proteomics

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

For accurate high-throughput systems biology approaches, chemostat cultures present many advantages (see Box 2); thus, in many large-scale studies, chemostats were generally chosen as cultivation systems to provide reasonably comparable data [49]. The first combined omics study on *C. acetobutylicum* under steady-state conditions was published in 2010 [50]. In this study, qualitative two-color microarray and 2-DE were carried out to analyze acidogenesis and solventogenesis; marked acidogenesis-specific expression of an operon comprising CA_P0036-37, which was disregarded in previous batch-condition investigations, was discovered under steady-state conditions.

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

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

Metabolomics/Fluxomics

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

Quantitative omics approaches in physiological studies and metabolic engineering

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

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

A few studies have focused on the analysis of *C. acetobutylicum* physiology in steady-state chemostat cultures under acidogenic, solventogenic or alcohologenic conditions. By analyzing quantitative transcriptomic, proteomic, fluxomic and biochemical data, Yoo et al. elucidated the activity distributions of the five enzymes (AdhE1, AdhE2, BdhA, BdhB and BdhC) involved in the two-step butanol pathway (Figure 1) [17]. Under solventogenic conditions, the first step was performed mainly by the NADH-dependent aldehyde dehydrogenase activity of AdhE1, whereas the second step was performed by the NADPH-dependent alcohol dehydrogenase activity of BdhB, as AdhE1 possesses very low alcohol dehydrogenase activity. On the other hand, under alcohologenic conditions, AdhE2 was responsible for NADH-dependent aldehyde and alcohol dehydrogenase activities. When *adhE1* was deleted, *adhE2* was expressed under solventogenic conditions, and AdhE2 could replace AdhE1 to catalyze the conversion of butyryl-CoA to butyraldehyde [51]. In contrast, when *adhE2* was deleted, AdhE1 could not replace AdhE2 under any of the metabolic conditions [51]. The roles of the two homologous *buk* (or *buk1*) and *buk2* genes were also elucidated using the same approach [52]. Buk has been reported to be the major butyrate kinase [52, 59]. Additionally, deletion of *buk* could not be achieved without (partial) disruption of the *ptb* gene, most likely due to the toxicity of butyryl phosphate accumulation (Figure 1). Interestingly, AdhE2 was highly expressed in a *ΔbukΔptb* mutant under all steady-state metabolic conditions; a high butanol yield was obtained, and lactate was the main product in alcohologenesis [52]. Using a similar approach, CA_P0037, a global regulator of central metabolism, was characterized by Nguyen et al. [53]. The metabolic

flux in acidogenesis and alcohologenesis changed significantly when CA_P0037 was disrupted. Acids (lactate, acetate and butyrate) were the major products in alcohologenesis, whereas butanol and lactate levels increased in acidogenesis. These behaviors were consistent with the quantitative transcriptomic and proteomic profiles.

There have been many studies on the behavior of *C. acetobutylicum* cells under butanol stress that have used transcriptomic data alone [28, 35, 60, 61] or in combination with proteomic [46] and fluxomic [23] data (Table 1). COnstraint-Based Reconstruction and Analysis (COBRA, metabolic modeling framework applying different constrains limiting achievable cellular function [62]) with additional constraints obtained from ^{13}C-MFA [63] was used to quantitatively analyze the effects of butanol stress on cellular metabolism i.e. low yield of biomass per ATP, slight effect on glycolysis but more on TCA cycle and serine/glycine pathway [64]. Via a metabolomics-based approach to investigate the intracellular biochemical changes in cells treated with butanol under acidogenic or solventogenic conditions, Wang et al. [65] demonstrated that i) a more active bifurcated TCA cycle, ii) an increased glycerol and amino acid formation, and iii) an altered fatty acid composition were key factors that contributed to the butanol tolerance of the cells. The same metabolomic approach was also employed to determine the key metabolites that might affect cell growth and butanol production [66]. Sugar metabolism and amino acid metabolism are favorable for cell growth and unfavorable for butanol production. Based on an analysis of dicarboxylate metabolism, ethylene glycol and citric acid supplementation during fermentation increased the formation of intermediates that promoted both cell growth and butanol production.

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

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

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

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

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

Regarding a more recent study, a *C. acetobutylicum* mutant was engineered to continuously produce *n*-butanol at very high yields with very high selectivity and productivity [9]. The straightforward engineering process was guided by i) fluxomics analysis using the GSM *i*Cac967 (which was developed by the same group) and the accurate calculation of all the electron fluxes [17] and ii) transcriptomics, proteomics and fluxomics analysis of a butyrate minus mutant [52]. This strain contained multiple deletions, including genes encoding for competing pathways such as butyrate formation (*ΔptbΔbuk*), lactate formation (*Δldh*), acetone formation (*ΔctfAB*), and for a redox-

sensing transcriptional regulator (*ΔrexA*) that had been known to repress genes involved in the C4 formation pathway (*thlA, crt-bcd-etfAB-hbd* and *adhE2*). In addition to those deletions, gene substitutions *ΔthlA::atoB* and *Δhbd::hbd1* provided enzymes with improved characteristics that were effective at pulling the carbon flux toward the formation of butanol by making use of the high NADPH/$NADP^+$ ratio driving force. The strain obtained produced butanol at 83% of the maximum theoretical yield (Figure 3d). The butanol-to-ethanol ratio increased 6-fold. However, the acetate formation pathway was still competing for carbon flux, as the strain produced acetate at low levels [9]. The intensive attempt to abolish all acid formation pathways, including acetate, was unsuccessful. Thus far, eliminating all by-products completely in *C. acetobutylicum* is not possible. Currently, the GSM *i*Cac967 is unable to explain this phenomenon. More studies must be performed to understand the physiology and regulatory systems prior to incorporating this knowledge into cell metabolic models for further strain engineering.

Concluding Remarks and Future Perspectives

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

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

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

Box 1. Physiological characteristics of *Clostridium acetobutylicum*

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

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

Box 2. Advantages of chemostat cultures for systems biology studies

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

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

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

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

hydroxybutyryl-CoA dehydrogenase from *Clostridium kluyveri*; p_{ptb}-*alsD*-$_{RBS}$*bdhA*-$_{RBS}$*acr,* plasmid expressing genes from the *ptb* promoter*;* p_{ptb}*adhE1*^{D485G}, plasmid expressing a mutated (D485G) Adhe1; pSOL1: megaplasmid of *C. acetobutylicum* (a Δ*pSOL1* strain is unable to produce solvents and spores); pSPD5*(dhaB1 dhaB2 dhaT),* plasmid expressing genes encoding a glycerol dehydratase (*dhaB1,dhaB2)* and 1,3-propanediol dehydrogenase (*dhaT*)

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

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

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

Category	Omics	Technique/Culture type/Strain	Main conclusion	Remarks	References
i) Analysis of different metabolic states or transition states from acidogenesis to solventogenesis					
	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 *C. acetobutylicum* 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, p*I* 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 *C. acetobutylicum.*	[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) & ^{1}H-nuclear magnetic resonance (^{1}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 *adhE1/2* and *thlA/B*, 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 *adhE2* and repressed *sol* operon (*adhE1, ctfAB*), as well as *adc*	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 *adhE2* in contrast to low *adhE1* expression.	[74]
	Transcriptomics	DNA Microarray/Batch/*sigG* (Sporulation-Specific Sigma Factor G) mutant	σK is necessary in early sporulation prior to *spo0A* expression and is also necessary in late sporulation. New sporulation cascade model proposed	Study of the *sigG* 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)/C. *acetobutylicum* 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 *C. acetobutylicum*, only relative fold changes presented.	[83]
ii) Analysis of metabolite stress, such as butanol/butyrate stress					
	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 *groESL* operon (involved in regulation of heat shock gene expression).	[35]
	Transcriptomics	DNA Microarray/Batch (butanol stress-induced flask or bioreactor)/*spo0A* (the regulator of endospore formation) -overexpressing vs knockout strains	Overexpression of *spo0A* 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 *spo0A* and comparison to spo0A 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 *C. acetobutylicum*	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]
iii) Utilization of different substrates					
	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 ThlA in stationary phase was reported, while gene products of *hbd*, *crt* and *bcd* were not reported as significantly changed; unfortunately metabolite profiles were not included for further analysis.	[87]
	Transcriptomics	DNA microarray/Batch (glucose + xylose)/*ccpA*-inactivated strain vs WT	CcpA plays a role in carbon catabolite repression, inactivation of *ccpA* influenced solvent production genes (upregulation of *sol* operon) as well as sporulation genes (*ccpA* is necessary for efficient sporulation)	Transcriptionally affected genes, in particular in carbohydrate metabolism, by inactivation of *ccpA* encoding catabolite control protein.	[88]
	Fluxomics and metabolomics	Gas chromatography mass spectrometry (GC-MS)/*xfp* (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. *xfp*-inactivated mutant still had phosphoketolase activity; on the	Use of ^{13}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 (^{1}H NMR) and liquid chromatography mass spectrometry (LC-MS)/Batch (glucose, glucose + xylose, glucose + arabinose, and xylose + arabinose)/WT	Hierarchy in *C. acetobutylicum* 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 $NADP^+$-to-NADPH ratio detected may be related to solvent-specific productivity, which was not affected	A recent quantitative metabolomics characterization of *C. acetobutylicum* grown on xylose with a high concentration of sodium to understand high sodium-mediated inhibition of cell proliferation but similar solvent productivity.	[90]
iv) Key metabolic mutants versus the wild-type strain					
	Transcriptomics	DNA microarray (1,019 genes including all 178 pSOL1 genes)/Batch/*spo0A*-	The majority of gene expression patterns in SKO1 are similar to those in *B. subtilis*	The first omics study of *C. acetobutylicum* 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 *sigF* (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 *C. acetobutylicum* and *B. subtilis* (cf. Until this study, transcript-level understanding relied only on studies in *Bacillus subtilis*, 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 *C. acetobutylicum* 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)/Δ*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 *perR* (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 *adhE2*, hydrogenase gene (*hydA*), and *spo0A*, 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/*fur*-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 *fur* altered expression of *ldhA* and the main flavodoxin-encoding gene	Wild-type and *fur* (ferric uptake regulator) mutant grown under iron-repleted and depleted conditions were analyzed by transcriptomic and proteomic approaches.	[96]
	Transcriptomics	DNA Microarray/Continuous/Δ*ptb,* Δ*pta,* Δ*adc,* Δ*adc*Δ*pta* strains	*ptb* 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]

			butanol as the major product in acidogenesis and solventogenesis) than that from the batch culture (ethanol as the major product) [97]. Strikingly upregulated *adhE2* detected in Δ*pta* and Δ*adc*Δ*pta* under both metabolic conditions. However, in Δ*ptb*, only slightly increased expression of this gene in acidogenesis was claimed, which does not agree with [52], which showed high expression of the gene	changes under acidogenesis and solventogenesis	
	Transcriptomics, proteomics and fluxomics	DNA microarray/Continuous culture/Δ*adhE1*, Δ*adhE2* strains	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	Elucidation of the roles of *adhE1* and *adhE2* in the primary metabolism of *C. acetobutylicum* using a quantitative systems biology approach.	[51]

	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 *ldhA* and downregulated *adhE2*. 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 *C. acetobutylicum* metabolism.	[53]
	Transcriptomics, proteomics and fluxomics	DNA microarray/Continuous/Δ*buk*Δ *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 *adhE2* detected in all metabolic conditions	Analysis of the metabolic flexibility of a butyrate pathway mutant of *C. acetobutylicum* using a quantitative systems biology approach.	[52]

	Transcriptomics and metabolomics	Liquid chromatography-high-resolution mass spectroscopy (LC-HRMS) and RNA-seq/Batch/Δ*pks*	Impacts of polyketide production in *C. acetobutylicum*: Slightly increased solvent production but no significant transcriptional change in the *sol* operon in Δ*pks*. The polyketides act like triggers of sporulation related to sigma K expression	In Δ*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 *in silico* metabolic flux analysis of this strain using genome-scale model *i*Cac802.	[99]
v) Others					
	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 *C. acetobutylicum*	[100]
	Proteomics	2-DE/Continuous/WT	Respective SOPs for culture supernatant and cell pellet for 2-DE of *C. acetobutylicum* 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	13C-based isotopomer analysis/Batch/WT	Although genome annotation of *C. acetobutylicum* lacked most corresponding TCA cycle genes, this study demonstrated a complete bifurcated TCA cycle. In detail, oxaloacetate flows to succinate both through citrate (clockwise)/α-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	13C-based isotopomer analysis/Batch/WT	Split TCA cycle where only Re-citrate synthase contributes to α-ketoglutarate production; the oxidative pentose-phosphate pathway (oxPPP) is inactive	Determination of central metabolism pathway, particularly the TCA cycle, in *C. acetobutylicum* 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 *C. acetobutylicum*. 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	13C-based isotopomer analysis/Batch/WT	In this study, no measurable flux between α-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 *C. acetobutylicum*	Metabolome methodology development for atmospheric metabolite sampling and processing of the strict anaerobe *C. acetobutylicum*.	[103]

Acknowledgments

MY, NPTN and PS acknowledge financial support from the European Community's Seventh Framework Program "CLOSTNET" (PEOPLE-ITN-2008-237942) and the Metabolic Explorer Company.

References

1. Bormann, S. et al. (2014) Engineering Clostridium acetobutylicum for production of kerosene and diesel blendstock precursors. Metab Eng 25, 124-30.
2. Lee, J. et al. (2012) Metabolic engineering of Clostridium acetobutylicum ATCC 824 for isopropanol-butanol-ethanol fermentation. Appl Environ Microbiol 78 (5), 1416-23.
3. Dusseaux, S. et al. (2013) Metabolic engineering of Clostridium acetobutylicum ATCC 824 for the high-yield production of a biofuel composed of an isopropanol/butanol/ethanol mixture. Metab Eng 18, 1-8.
4. Jang, Y.S. et al. (2013) Metabolic engineering of Clostridium acetobutylicum for the enhanced production of isopropanol-butanol-ethanol fuel mixture. Biotechnol Prog 29 (4), 1083-8.
5. Bankar, S.B. et al. (2015) Genetic engineering of Clostridium acetobutylicum to enhance isopropanol-butanol-ethanol production with an integrated DNA-technology approach. Renew Energy 83, 1076-1083.
6. Wang, C. et al. (2018) Enhanced isopropanol-butanol-ethanol mixture production through manipulation of intracellular NAD(P)H level in the recombinant Clostridium acetobutylicum XY16. Biotechnol Biofuels 11, 12.
7. Liu, D. et al. (2018) Towards acetone-uncoupled biofuels production in solventogenic Clostridium through reducing power conservation. Metab Eng 47, 102-112.
8. Nicolaou, S.A. et al. (2010) A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: From biofuels and chemicals, to biocatalysis and bioremediation. Metab Eng 12 (4), 307-331.
9. Nguyen, N.P. et al. (2018) Reviving the Weizmann process for commercial n-butanol production. Nat Commun 9, 3682.
10. Gonzalez-Pajuelo, M. et al. (2006) Microbial conversion of glycerol to 1,3-propanediol: Physiological comparison of a natural producer, Clostridium butyricum VPI 3266, and an engineered strain, Clostridium acetobutylicum DG1(pSPD5). Appl Environ Microbiol 72 (1), 96-101.
11. Collas, F. et al. (2012) Simultaneous production of isopropanol, butanol, ethanol and 2,3-butanediol by Clostridium acetobutylicum ATCC 824 engineered strains. Amb Express 2, 45.
12. Jang, Y.S. et al. (2014) Metabolic engineering of Clostridium acetobutylicum for butyric acid production with high butyric acid selectivity. Metab Eng 23, 165-174.
13. Millat, T. and Winzer, K. (2017) Mathematical modelling of clostridial acetone-butanol-ethanol fermentation. Appl Microbiol Biotechnol 101 (6), 2251-2271.
14. Liao, C. et al. (2015) Integrated, systems metabolic picture of acetone-butanol-ethanol fermentation by Clostridium acetobutylicum. Proc Natl Acad Sci U S A 112 (27), 8505-8510.
15. Joseph, R.C. et al. (2018) Recent developments of the synthetic biology toolkit for Clostridium. Front Microbiol 9, 154.

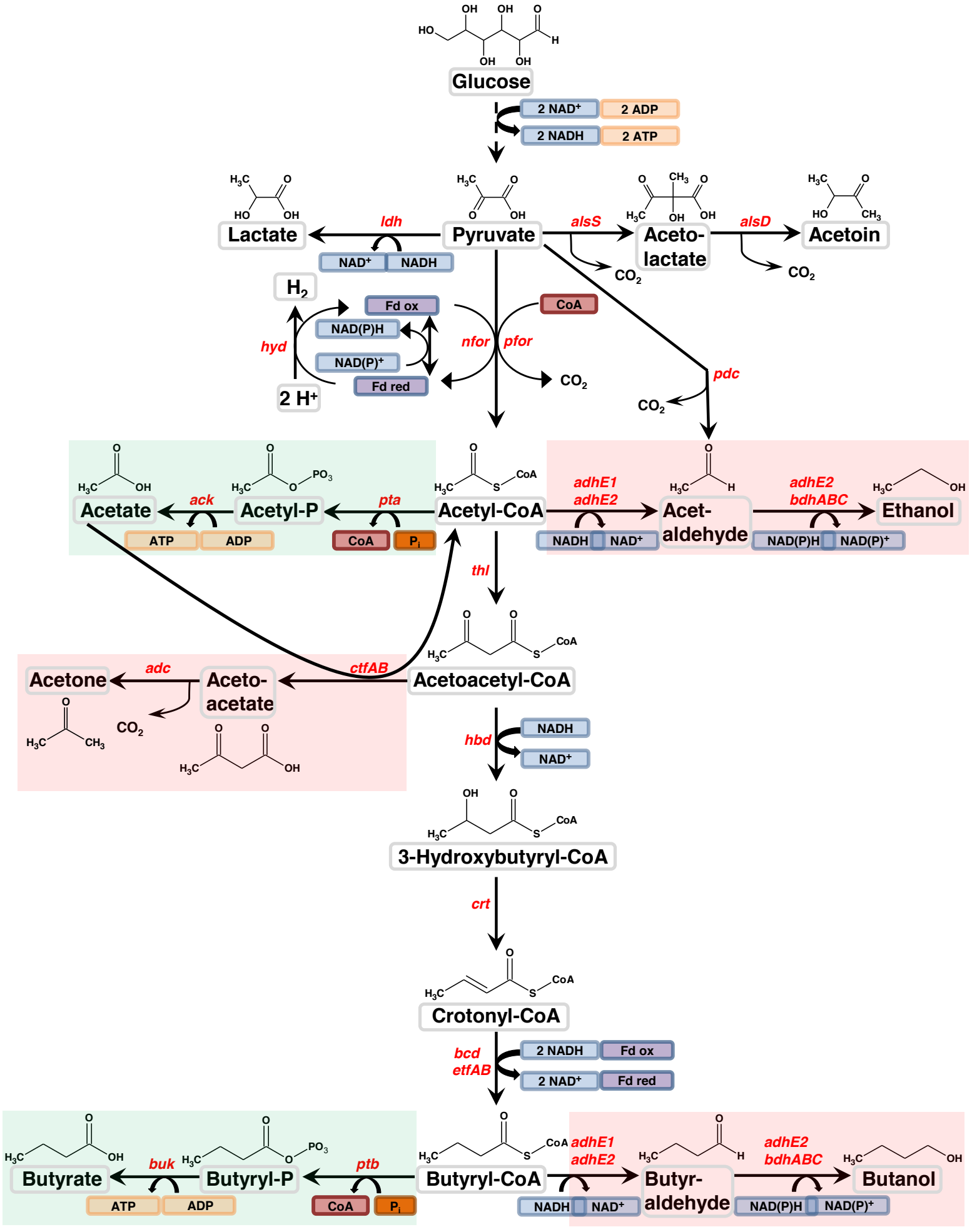
16. Senger, R.S. and Papoutsakis, E.T. (2008) Genome-scale model for Clostridium acetobutylicum: Part I. Metabolic network resolution and analysis. Biotechnol Bioeng 101 (5), 1036-52.
17. Yoo, M. et al. (2015) A quantitative system-scale characterization of the metabolism of Clostridium acetobutylicum. mBio 6 (6), e01808-15.
18. Girbal, L. and Soucaille, P. (1994) Regulation of Clostridium acetobutylicum metabolism as revealed by mixed-substrate steady-state continuous cultures: role of NADH/NAD ratio and ATP pool. J Bacteriol 176 (21), 6433-6438.
19. Thiele, I. and Palsson, B.O. (2010) A protocol for generating a high-quality genome-scale metabolic reconstruction. Nat Protoc 5 (1), 93-121.
20. Lee, J. et al. (2008) Genome-scale reconstruction and in silico analysis of the Clostridium acetobutylicum ATCC 824 metabolic network. Appl Microbiol Biotechnol 80 (5), 849-62.
21. Senger, R.S. and Papoutsakis, E.T. (2008) Genome-scale model for Clostridium acetobutylicum: Part II. Development of specific proton flux states and numerically determined sub-systems. Biotechnol Bioeng 101 (5), 1053-71.
22. McAnulty, M.J. et al. (2012) Genome-scale modeling using flux ratio constraints to enable metabolic engineering of clostridial metabolism in silico. BMC Syst Biol 6, 42.
23. Dash, S. et al. (2014) Capturing the response of Clostridium acetobutylicum to chemical stressors using a regulated genome-scale metabolic model. Biotechnol Biofuels 7, 144.
24. Salimi, F. et al. (2010) Genome-scale metabolic modeling of a clostridial co-culture for consolidated bioprocessing. Biotechnol J 5 (7), 726-738.
25. Wallenius, J. et al. (2013) Constraint-based genome-scale metabolic modeling of Clostridium acetobutylicum behavior in an immobilized column. Bioresour Technol 142, 603-610.
26. Amador-Noguez, D. et al. (2011) Metabolome remodeling during the acidogenic-solventogenic transition in Clostridium acetobutylicum. Appl Environ Microbiol 77 (22), 7984-97.
27. Wang, Q. et al. (2013) Transcription factors and genetic circuits orchestrating the complex, multilayered response of Clostridium acetobutylicum to butanol and butyrate stress. BMC Syst Biol 7, 120.
28. Alsaker, K.V. et al. (2010) Metabolite stress and tolerance in the production of biofuels and chemicals: gene-expression-based systems analysis of butanol, butyrate, and acetate stresses in the anaerobe Clostridium acetobutylicum. Biotechnol Bioeng 105 (6), 1131-47.
29. Nolling, J. et al. (2001) Genome sequence and comparative analysis of the solvent-producing bacterium Clostridium acetobutylicum. J Bacteriol 183 (16), 4823-38.
30. Tomas, C.A. et al. (2003) DNA array-based transcriptional analysis of asporogenous, nonsolventogenic Clostridium acetobutylicum strains SKO1 and M5. J Bacteriol 185 (15), 4539-47.
31. Yang, H. et al. (2003) A segmental nearest neighbor normalization and gene identification method gives superior results for DNA-array analysis. Proc Natl Acad Sci U S A 100 (3), 1122-7.
32. Tomas, C.A. et al. (2003) Overexpression of groESL in Clostridium acetobutylicum results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. Appl Environ Microbiol 69 (8), 4951-65.
33. Tummala, S.B. et al. (2003) Antisense RNA downregulation of coenzyme A transferase combined with alcohol-aldehyde dehydrogenase overexpression leads to predominantly alcohologenic Clostridium acetobutylicum fermentations. J Bacteriol 185 (12), 3644-53.

538 34. Tummala, S.B. et al. (2003) Transcriptional analysis of product-concentration driven
539 changes in cellular programs of recombinant Clostridium acetobutylicumstrains. Biotechnol
540 Bioeng 84 (7), 842-54.
541 35. Tomas, C.A. et al. (2004) Transcriptional analysis of butanol stress and tolerance in
542 Clostridium acetobutylicum. J Bacteriol 186 (7), 2006-18.
543 36. Alsaker, K.V. et al. (2004) Transcriptional analysis of spo0A overexpression in Clostridium
544 acetobutylicum and its effect on the cell's response to butanol stress. J Bacteriol 186 (7), 1959-
545 1971.
546 37. Zhao, Y. et al. (2005) Intracellular butyryl phosphate and acetyl phosphate concentrations
547 in Clostridium acetobutylicum and their implications for solvent formation. Appl Environ
548 Microbiol 71 (1), 530-7.
549 38. Sullivan, L. et al. (2007) Analysis of the clostridial hydrophobic with a conserved tryptophan
550 family (ChW) of proteins in Clostridium acetobutylicum with emphasis on ChW14 and
551 ChW16/17. Enzyme Microb Technol 42 (1), 29-43.
552 39. Hu, S.Y. et al. (2011) Comparative genomic and transcriptomic analysis revealed genetic
553 characteristics related to solvent formation and xylose utilization in Clostridium
554 acetobutylicum EA 2018. BMC Genomics 12, 93.
555 40. Servinsky, M.D. et al. (2010) Transcriptional analysis of differential carbohydrate utilization
556 by Clostridium acetobutylicum. Microbiology 156, 3478-91.
557 41. Cornillot, E. et al. (1997) The genes for butanol and acetone formation in Clostridium
558 acetobutylicum ATCC 824 reside on a large plasmid whose loss leads to degeneration of the
559 strain. J Bacteriol 179 (17), 5442-7.
560 42. Alsaker, K.V. and Papoutsakis, E.T. (2005) Transcriptional program of early sporulation and
561 stationary-phase events in Clostridium acetobutylicum. J Bacteriol 187 (20), 7103-18.
562 43. Venkataramanan, K.P. et al. (2013) The Clostridium small RNome that responds to stress:
563 the paradigm and importance of toxic metabolite stress in C. acetobutylicum. BMC Genomics
564 14, 849.
565 44. Ralston, M.T. and Papoutsakis, E.T. (2018) RNAseq-based transcriptome assembly of
566 Clostridium acetobutylicum for functional genome annotation and discovery. AIChE J
567 doi:10.1002/aic.16396.
568 45. Servinsky, M.D. et al. (2018) Arabinose-induced catabolite repression as a mechanism for
569 pentose hierarchy control in Clostridium acetobutylicum ATCC 824. mSystems 3 (5), e00064-
570 18.
571 46. Venkataramanan, K.P. et al. (2015) Complex and extensive post-transcriptional regulation
572 revealed by integrative proteomic and transcriptomic analysis of metabolite stress response
573 in Clostridium acetobutylicum. Biotechnol Biofuels 8, 81.
574 47. Schaffer, S. et al. (2002) Changes in protein synthesis and identification of proteins
575 specifically induced during solventogenesis in Clostridium acetobutylicum. Electrophoresis 23
576 (1), 110-121.
577 48. Sullivan, L. and Bennett, G.N. (2006) Proteome analysis and comparison of Clostridium
578 acetobutylicum ATCC 824 and Spo0A strain variants. J Ind Microbiol Biotechnol 33 (4), 298-
579 308.
580 49. Sauer, U. (2004) High-throughput phenomics: experimental methods for mapping
581 fluxomes. Curr Opin Biotechnol 15 (1), 58-63.
582 50. Janssen, H. et al. (2010) A proteomic and transcriptional view of acidogenic and
583 solventogenic steady-state cells of Clostridium acetobutylicum in a chemostat culture. Appl
584 Microbiol Biotechnol 87 (6), 2209-2226.

585 51. Yoo, M. et al. (2016) Elucidation of the roles of adhE1 and adhE2 in the primary metabolism
586 of Clostridium acetobutylicum by combining in-frame gene deletion and a quantitative
587 system-scale approach. Biotechnol Biofuels 9, 92.
588 52. Yoo, M. et al. (2017) Metabolic flexibility of a butyrate pathway mutant of Clostridium
589 acetobutylicum. Metab Eng 40, 138-147.
590 53. Nguyen, N.P. et al. (2016) Cap0037, a Novel Global Regulator of Clostridium
591 acetobutylicum Metabolism. mBio 7 (5), e01218-16.
592 54. Crown, S.B. et al. (2011) Resolving the TCA cycle and pentose-phosphate pathway of
593 Clostridium acetobutylicum ATCC 824: Isotopomer analysis, in vitro activities and expression
594 analysis. Biotechnol J 6 (3), 300-5.
595 55. Amador-Noguez, D. et al. (2010) Systems-level metabolic flux profiling elucidates a
596 complete, bifurcated tricarboxylic acid cycle in Clostridium acetobutylicum. J Bacteriol 192
597 (17), 4452-61.
598 56. Au, J. et al. (2014) Parallel labeling experiments validate Clostridium acetobutylicum
599 metabolic network model for (13)C metabolic flux analysis. Metab Eng 26, 23-33.
600 57. Aristilde, L. et al. (2015) Hierarchy in pentose sugar metabolism in Clostridium
601 acetobutylicum. Appl Environ Microbiol 81 (4), 1452-62.
602 58. Aristilde, L. (2017) Metabolite labelling reveals hierarchies in Clostridium acetobutylicum
603 that selectively channel carbons from sugar mixtures towards biofuel precursors. Microb
604 Biotechnol 10 (1), 162-174.
605 59. Huang, K.X. et al. (2000) Identification and characterization of a second butyrate kinase
606 from Clostridium acetobutylicum ATCC 824. J Mol Microbiol Biotechnol 2 (1), 33-8.
607 60. Janssen, H. et al. (2012) A transcriptional study of acidogenic chemostat cells of
608 Clostridium acetobutylicum--solvent stress caused by a transient n-butanol pulse. J Biotechnol
609 161 (3), 354-65.
610 61. Wang, Q.H. et al. (2013) Transcription factors and genetic circuits orchestrating the
611 complex, multilayered response of Clostridium acetobutylicum to butanol and butyrate stress.
612 BMC Syst Biol 7 (120).
613 62. Price, N.D. et al. (2004) Genome-scale models of microbial cells: Evaluating the
614 consequences of constraints. Nat Rev Microbiol 2 (11), 886-897.
615 63. Wiechert, W. (2001) C-13 metabolic flux analysis. Metab Eng 3, 195-206.
616 64. Wallenius, J. et al. (2016) Carbon 13-Metabolic Flux Analysis derived constraint-based
617 metabolic modelling of Clostridium acetobutylicum in stressed chemostat conditions.
618 Bioresour Technol 219, 378-386.
619 65. Wang, Y.F. et al. (2016) Intracellular metabolic changes of Clostridium acetobutylicum and
620 promotion to butanol tolerance during biobutanol fermentation. Int J Biochem Cell Biol 78,
621 297-306.
622 66. Liu, H. et al. (2016) Integrated intracellular metabolic profiling and pathway analysis
623 approaches reveal complex metabolic regulation by Clostridium acetobutylicum. Microb Cell
624 Fact 15, 36.
625 67. Herman, N.A. et al. (2017) The industrial anaerobe Clostridium acetobutylicum uses
626 polyketides to regulate cellular differentiation. Nat commun 8, 1514.
627 68. Jang, Y.S. et al. (2012) Enhanced butanol production obtained by reinforcing the direct
628 butanol-forming route in Clostridium acetobutylicum. mBio 3 (5), e00314-12.
629 69. Vasconcelos, I. et al. (1994) Regulation of carbon and electron flow in Clostridium
630 acetobutylicum grown in chemostat culture at neutral pH on mixtures of glucose and glycerol.
631 J Bacteriol 176 (5), 1443-1450.

70. Raynaud, C. et al. (2003) Molecular characterization of the 1,3-propanediol (1,3-PD) operon of Clostridium butyricum. Proc Natl Acad Sci U S A 100 (9), 5010-5015.
71. Gonzalez-Pajuelo, M. et al. (2005) Metabolic engineering of Clostridium acetobutylicum for the industrial production of 1,3-propanediol from glycerol. Metab Eng 7 (5-6), 329-336.
72. Grupe, H. and Gottschalk, G. (1992) Physiological events in Clostridium acetobutylicum during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. Appl Environ Microbiol 58 (12), 3896-3902.
73. Rao, G. and Mutharasan, R. (1986) Alcohol production by Clostridium acetobutylicum induced by methyl viologen. Biotechnol Lett 8 (12), 893-896.
74. Honicke, D. et al. (2012) Global transcriptional changes of Clostridium acetobutylicum cultures with increased butanol:acetone ratios. N Biotechnol 29 (4), 485-93.
75. Davidi, D. et al. (2016) Global characterization of in vivo enzyme catalytic rates and their correspondence to in vitro k(cat) measurements. Proc Natl Acad Sci U S A 113 (12), 3401-3406.
76. O'Brien, E.J. and Palsson, B.O. (2015) Computing the functional proteome: recent progress and future prospects for genome-scale models. Curr Opin Biotechnol 34, 125-134.
77. Papoutsakis, E.T. (1984) Equations and calculations for fermentations of butyric acid bacteria. Biotechnol Bioeng 26 (2), 174-187.
78. Haus, S. et al. (2011) A systems biology approach to investigate the effect of pH-induced gene regulation on solvent production by Clostridium acetobutylicum in continuous culture. BMC Syst Biol 5, 10.
79. Grimmler, C. et al. (2011) Genome-wide gene expression analysis of the switch between acidogenesis and solventogenesis in continuous cultures of Clostridium acetobutylicum. J Mol Microbiol Biotechnol 20 (1), 1-15.
80. Peguin, S. and Soucaille, P. (1995) Modulation of carbon and electron flow in Clostridium acetobutylicum by iron limitation and methyl viologen addition. Appl Environ Microbiol 61 (1), 403-405.
81. Al-Hinai, M.A. et al. (2014) sigmaK of Clostridium acetobutylicum is the first known sporulation-specific sigma factor with two developmentally separated roles, one early and one late in sporulation. J Bacteriol 196 (2), 287-99.
82. Liu, D. et al. (2013) Enhanced butanol production by modulation of electron flow in Clostridium acetobutylicum B3 immobilized by surface adsorption. Bioresour Technol 129, 321-328.
83. Liu, D. et al. (2016) Comparative transcriptomic analysis of Clostridium acetobutylicum biofilm and planktonic cells. J Biotechnol 218, 1-12.
84. Chen, Y.L. et al. (2011) Small RNAs in the genus Clostridium. mBio 2 (1), e00340-10.
85. Grimmler, C. et al. (2010) Transcriptional analysis of catabolite repression in Clostridium acetobutylicum growing on mixtures of D-glucose and D-xylose. J Biotechnol 150 (3), 315-23.
86. Sivagnanam, K. et al. (2011) Comparative shotgun proteomic analysis of Clostridium acetobutylicum from butanol fermentation using glucose and xylose. Proteome Sci 9, 66.
87. Sivagnanam, K. et al. (2012) Shotgun proteomic monitoring of Clostridium acetobutylicum during stationary phase of butanol fermentation using xylose and comparison with the exponential phase. J Ind Microbiol Biotechnol 39 (6), 949-55.
88. Ren, C. et al. (2012) Pleiotropic functions of catabolite control protein CcpA in Butanol-producing Clostridium acetobutylicum. BMC Genomics 13, 349.
89. Liu, L. et al. (2012) Phosphoketolase pathway for xylose catabolism in Clostridium acetobutylicum revealed by 13C metabolic flux analysis. J Bacteriol 194 (19), 5413-22.

678 90. Zhao, X. et al. (2016) A quantitative metabolomics study of high sodium response in
679 Clostridium acetobutylicum ATCC 824 acetone-butanol-ethanol (ABE) fermentation. Sci Rep
680 6, 28307.
681 91. Hillmann, F. et al. (2009) The role of PerR in O2-affected gene expression of Clostridium
682 acetobutylicum. J Bacteriol 191 (19), 6082-93.
683 92. Wang, S. et al. (2011) Formic acid triggers the "Acid Crash" of acetone-butanol-ethanol
684 fermentation by Clostridium acetobutylicum. Appl Environ Microbiol 77 (5), 1674-80.
685 93. Mao, S. et al. (2011) Comparative analysis on the membrane proteome of Clostridium
686 acetobutylicum wild type strain and its butanol-tolerant mutant. Mol Biosyst 7 (5), 1660-77.
687 94. Jia, K. et al. (2012) Identification and characterization of two functionally unknown genes
688 involved in butanol tolerance of Clostridium acetobutylicum. PLoS One 7 (6), e38815.
689 95. Mao, S. et al. (2010) Proteome reference map and comparative proteomic analysis
690 between a wild type Clostridium acetobutylicum DSM 1731 and its mutant with enhanced
691 butanol tolerance and butanol yield. J Proteome Res 9 (6), 3046-61.
692 96. Vasileva, D. et al. (2012) Effect of iron limitation and fur gene inactivation on the
693 transcriptional profile of the strict anaerobe Clostridium acetobutylicum. Microbiology 158,
694 1918-29.
695 97. Cooksley, C.M. et al. (2012) Targeted mutagenesis of the Clostridium acetobutylicum
696 acetone-butanol-ethanol fermentation pathway. Metab Eng 14 (6), 630-641.
697 98. Honicke, D. et al. (2014) Chemostat cultivation and transcriptional analyses of Clostridium
698 acetobutylicum mutants with defects in the acid and acetone biosynthetic pathways. Appl
699 Microbiol Biotechnol 98 (23), 9777-94.
700 99. Cho, C. et al. (2017) Genome analysis of a hyper acetone-butanol-ethanol (ABE) producing
701 Clostridium acetobutylicum BKM19. Biotechnol J 12, 1600457.
702 100. Paredes, C.J. et al. (2007) A general framework for designing and validating oligomer-
703 based DNA microarrays and its a application to Clostridium acetobutylicum. Appl Environ
704 Microbiol 73 (14), 4631-4638.
705 101. Schwarz, K. et al. (2007) A Standard Operating Procedure (SOP) for the preparation of
706 intra- and extracellular proteins of Clostridium acetobutylicum for proteome analysis. J
707 Microbiol Methods 68 (2), 396-402.
708 102. Hou, S. et al. (2013) Workflow for quantitative proteomic analysis of Clostridium
709 acetobutylicum ATCC 824 using iTRAQ tags. Methods 61 (3), 269-76.
710 103. Lee, S.H. et al. (2014) Atmospheric vs. anaerobic processing of metabolome samples for
711 the metabolite profiling of a strict anaerobic bacterium, Clostridium acetobutylicum.
712 Biotechnol Bioeng 111 (12), 2528-2536.
713


Glucose
2 NAD+
2 ADP
2 NADH
2 ATP
Lactate
ldh
NAD+
NADH
Pyruvate
alsS
CO2
Aceto-lactate
alsD
CO2
Acetoin
H2
hyd
2 H+
Fd ox
NAD(P)H
NAD(P)+
Fd red
nfor
pfor
CoA
CO2
pdc
CO2
Acetate
ack
ATP
ADP
Acetyl-P
pta
CoA
Pi
Acetyl-CoA
adhE1
adhE2
NADH
NAD+
Acet-aldehyde
adhE2
bdhABC
NAD(P)H
NAD(P)+
Ethanol
thl
Acetoacetyl-CoA
Acetone
adc
CO2
Aceto-acetate
ctfAB
hbd
NADH
NAD+
3-Hydroxybutyryl-CoA
crt
Crotonyl-CoA
bcd
etfAB
2 NADH
Fd ox
2 NAD+
Fd red
Butyrate
buk
ATP
ADP
Butyryl-P
ptb
CoA
Pi
Butyryl-CoA
adhE1
adhE2
NADH
NAD+
Butyr-aldehyde
adhE2
bdhABC
NAD(P)H
NAD(P)+
Butanol

[27, 37]

DNA

Genomics

Genome-sequencing

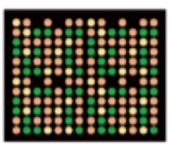
Transcription

RNA

Transcriptomics

Microarray

RNA-seq



[41, 97]

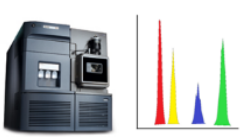
Translation

Protein

Proteomics

2-DE

LC/LC-MS/MS



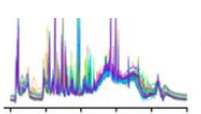
[17, 45]

Enzymatic reaction

Metabolite

Metabolomics

Isotopic labeling using GC/HPLC-MS

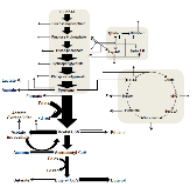


[53, 54]

Network

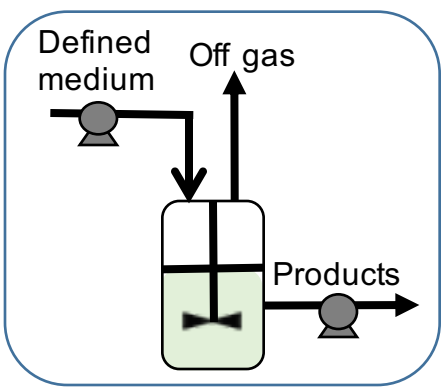
Flux

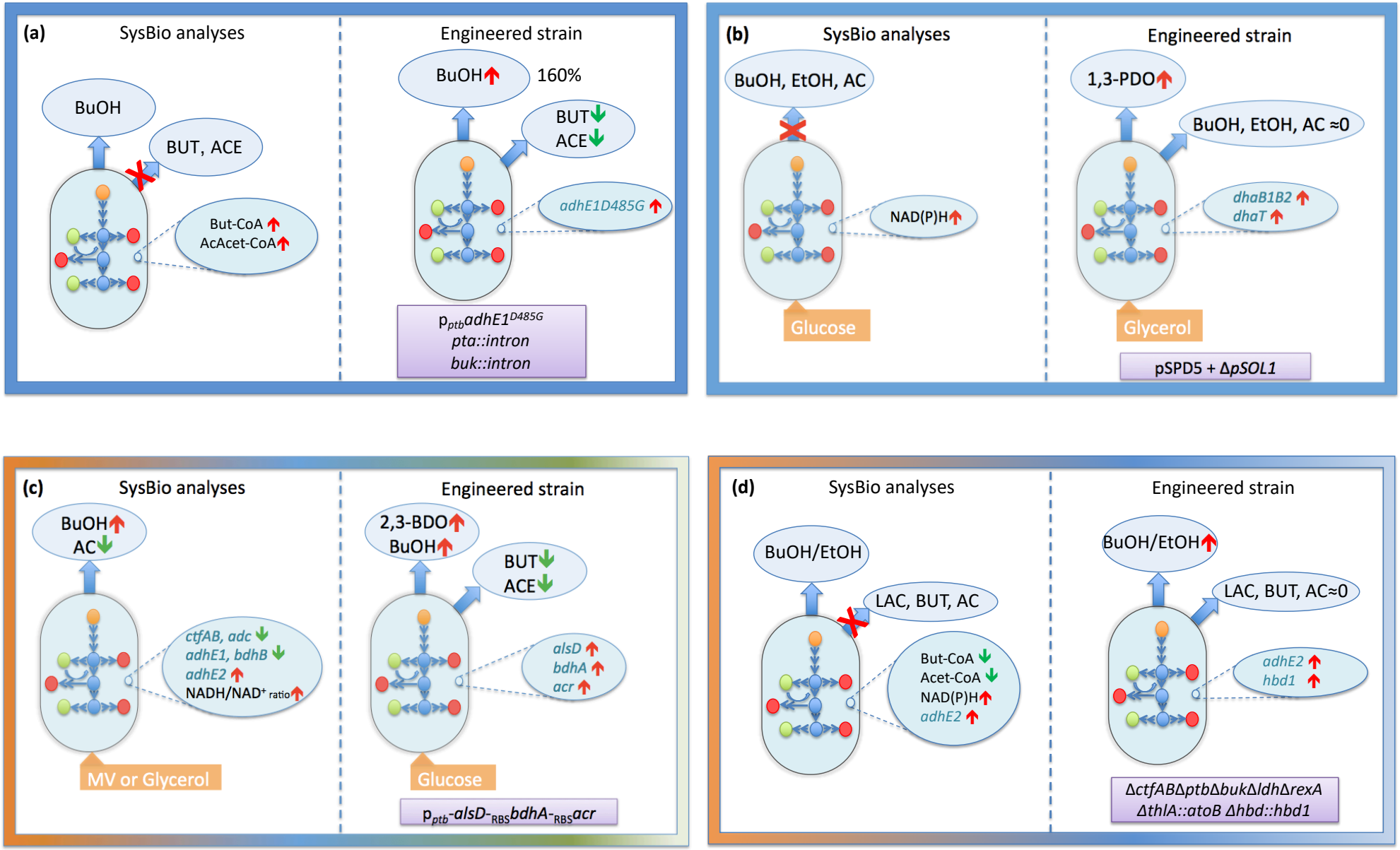
Fluxomics/GSM



[17, 75]

Physiological characterization/ Metabolic engineering




(a)
SysBio analyses
BuOH
BUT, ACE
But-CoA
AcAcet-CoA
Engineered strain
BuOH
160%
BUT
ACE
adhE1D485G
pptbadhE1D485G
pta::intron
buk::intron
(b)
SysBio analyses
BuOH, EtOH, AC
NAD(P)H
Glucose
Engineered strain
1,3-PDO
BuOH, EtOH, AC ≈0
dhaB1B2
dhaT
Glycerol
pSPD5 + ΔpSOL1
(c)
SysBio analyses
BuOH
AC
ctfAB, adc
adhE1, bdhB
adhE2
NADH/NAD+ ratio
MV or Glycerol
Engineered strain
2,3-BDO
BuOH
BUT
ACE
alsD
bdhA
acr
Glucose
ptb-alsD-RBSbdhA-RBSacr
(d)
SysBio analyses
BuOH/EtOH
LAC, BUT, AC
But-CoA
Acet-CoA
NAD(P)H
adhE2
Engineered strain
BuOH/EtOH
LAC, BUT, AC≈0
adhE2
hbd1
ΔctfABΔptbΔbukΔldhΔrexA
ΔthlA::atoB Δhbd::hbd1