

Engineering solventogenic clostridia

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Solventogenic clostridia are strictly anaerobic, endospore forming bacteria that produce a large array of primary metabolites, like butanol, by anaerobically degrading simple and complex carbohydrates, including cellulose and hemicellulose. Two genomes have been sequenced and some genetic tools have been developed, but more are now urgently needed. Genomic tools for designing, and assessing the impact of, genetic modifications are well developed. Early efforts to metabolically engineer these organisms suggest that they are promising organisms for biorefinery applications. Pathway engineering efforts have resulted in interesting strains, but global engineering of their transcriptional machinery has produced better outcomes. Future efforts are expected to undertake the development of complex multigenic phenotypes, such as aerotolerance, solvent tolerance, high-cell density fermentations, abolished sporulation without impacting product formation, and genetic stability for continuous bioprocessing.

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Introduction: solventogenic clostridia: from outcasts to workhorses?

The development of renewable chemicals and biofuel technologies has been on the scientific and technological agenda in the US and worldwide for over 35 years now, but never quite with the urgency and high priority of the past two years, when finally this technology has been elevated to a high priority status from being underappreciated (as many working in this field have experienced) by the broader scientific community, industry, and the funding agencies. Significantly, combustion of non-renewable fuels has increasingly apparent detrimental impact on the climate of our planet. Primary and waste biomass is a carbon-neutral renewable resource for the production of

biofuels, chemical, and materials, thus leading to the widely discussed concept of the biorefinery. Beyond ethanol, butanol is now emerging as an important biologically produced chemical and biofuel driven by its superior chemical properties. Butanol can be produced biologically by the anaerobic ABE (acetone-butanol-ethanol) clostridial fermentation [1], which was the primary source of butanol and acetone for over 40 years until the mid 1950s. Recently, there has been a renewed interest in the ABE fermentation, but butanol is only but one of the many chemicals that solventogenic clostridia could produce. Fortunately, as a result of several years of low key activity on the genetics and metabolic engineering of solventogenic clostridia (the main organisms being *Clostridium acetobutylicum* and *Clostridium beijerinckii*, both now sequenced), there are at least some tools available to do genomically-driven molecular engineering for strain development.

Clostridia are strictly anaerobic, endospore forming prokaryotes of major importance to cellulose degradation, human and animal health and physiology, anaerobic degradation of simple and complex carbohydrates, acidogenesis, and bioremediation of complex organics [2[•]]. Solventogenic, butyric acid clostridia can produce a large array of metabolites, and metabolic engineering (ME) driven strain development could enhance these native capabilities and may lead to industrial processes for production of additional (to butanol, acetone, and ethanol) chemicals including butyric and acetic acids, butanediol, propanol, and acetoin [1] and hydrogen [3]. Some of these chemicals can serve as biofuels directly, while others can be used for chemical conversion to biofuels (e.g. butyric acid [4^{••}]) or the generation of electricity [3]. Related clostridia can produce additional chemicals such as propionic and acrylic acids [5,6].

The potential of biorefinery/biofuel applications will not be fully realized until technologies are developed that would allow the utilization of cellulosic materials. In this context, a major advantage of solventogenic clostridia is their ability to utilize a large variety of carbon substrates [1]: monosaccharides, oligosaccharides, and polysaccharides, including refined and unrefined starches, many pentoses and hexoses, and as such to utilize biomass hydrolysates. Furthermore, many clostridia contain a complete or partial cellulosome (the complex biological system necessary for cellulose degradation) plus xylan-degradation enzymes, and may thus *directly* utilize cellulosic material for production of fuels and chemicals [7^{••}], all in a single fermentation step or sequential fermentation steps but using one type of organism.

Table 1

Summary of desirable metabolic engineering alterations of solventogenic clostridia and the potential impact they might have on bioprocessing

Strain or trait	Likely impact
Tolerate oxygen and possibly carrying out some aerobic metabolism	Simplify bioprocessing owing to less strict requirements for anaerobiosis; aerobic metabolism could enable higher cell densities without accumulating higher acid levels
Grow to much higher cell densities	Better volumetric productivity and faster completion of fermentation
Prolonged cell viability	Enhanced volumetric and cell specific productivity; prolonged productive fermentation; possibility of multi-cycle fed-batch fermentation
Direct utilization of cellulosics	Decreased substrate costs; possibly higher butanol tolerance owing to the impact of soluble and insoluble macromolecular carbohydrates
Asporogenous solvent-producing strains	Improved specific cell productivity and volumetric productivity; ability to use continuous or semi-continuous bioprocessing
Strains that do not degenerate (degeneration leads to reduced or no solvent production)	Ability to carry out multi-cycle fed-batch or continuous fermentations
Solvent tolerance	Higher solvent titers; improved cell-specific and volumetric productivity
Improved butanol selectivity (butanol becomes the sole or major solvent produced)	Better butanol yield per unit carbon substrate; simplified downstream processing; no undesirable side products

What is a target solvent productivity/titer for these fermentations to become economically attractive? There is no single number (total or butanol solvent titers, product yield, or productivity) that one can definitively offer as so much depends on the type of fermentation and process technology: batch, fed-batch, or continuous fermentation; the type of substrate used; if the process includes *in situ* product extraction; the technology used for product separation; energy recycle schemes, and so on. For traditional substrates, process design and economic issues have been recently reviewed [8^{*}], but there are no technologies yet available for fermentations based on cellulosic material. In the traditional batch process, butanol titers rarely exceeded 12–13 g/l. Assuming conventional substrates (molasses, corn-based starches, and related), economic analyses [9–12] have suggested that if the final butanol titers were raised from 12 to 19 g/l, the separation costs would be cut in half. These estimates are not, however, valid for cellulosic substrates, and neither are valid for processes where butanol is the only or the predominant product. We have generated strains [13,14] that produce up to 238 mM (17.6 g/l) butanol, 141 mM (8.2 g/l) acetone and 47 mM (2.2 g/l) ethanol (total solvents: 28 g/l). Blascheck's group has developed the *C. beijerickii* strain BA101, which produces high total solvent titers (up to 33 g/l) in batch fermentations, as reviewed [8^{*}]. Clearly, the field is wide open as to of the product characteristic targets for strain improvement by metabolic engineering. The review below is almost exclusively based on *C.*

acetobutylicum, the only organism whereby metabolic engineering tools have been employed for strain development.

Table 1 summarizes important strain characteristics/traits that could be developed employing metabolic engineering and the impact they might have on process improvements. These are discussed below in detail, but first we discuss important tools to enable metabolic engineering in these organisms.

The tools: to transform, overexpress, knockout (KO), knockdown (KD), to report, and to analyze *in vivo* fluxes, and the transcriptome

Although not as easy to employ, as reliable, and as fast as in well established model organisms (like *Escherichia coli* and *Bacillus subtilis*), solventogenic clostridia have now reasonably developed tools that have been recently reviewed, for example [15]. Briefly, transformation by electroporation and means to overcome the restriction system in *C. acetobutylicum* and plasmids to overexpress genes was published over 15 years ago [16], and this approach has been adapted to other clostridia. Methods for gene inactivation have been inefficient, first based on non-replicating vectors [17], and later using a replicating vector [18]. These methods are tedious, slow, and require substantial time and effort commitment. Most recently, the Targetron system (group II intron principle) was

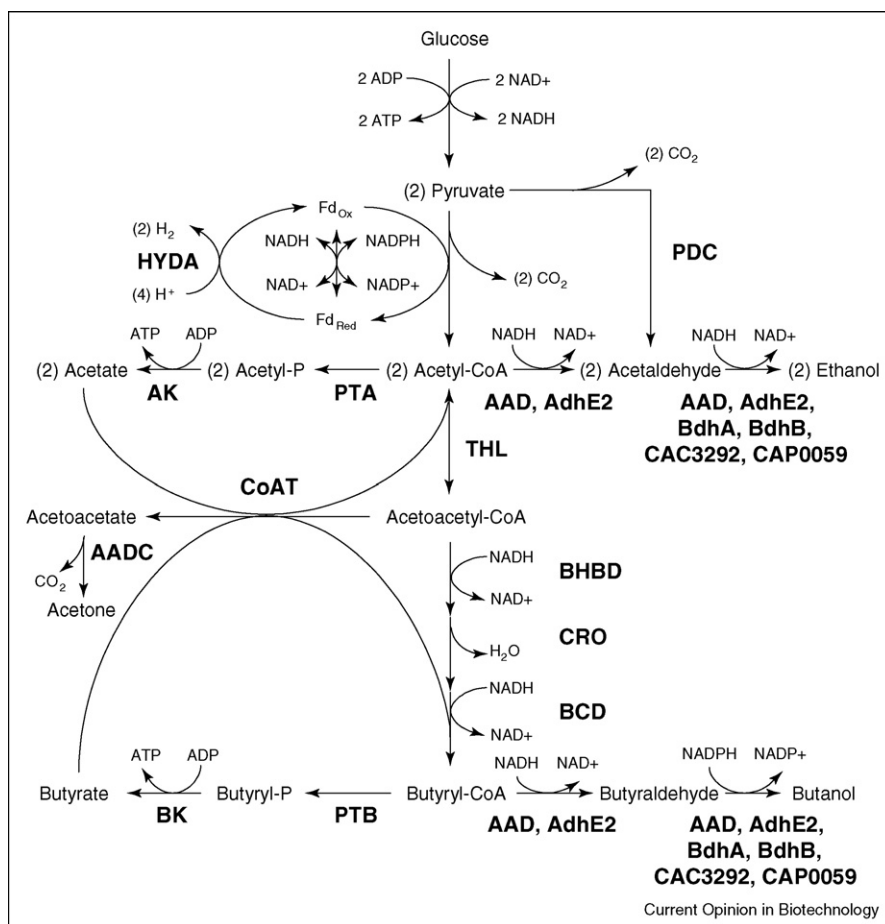
adapted to clostridia by two different groups [19^{••},20]. There are also two other recently reported methods. One is briefly discussed in the recent paper on the deletion of the (PerR)-homologous protein in *C. acetobutylicum*, and the other used to KO acid-formation genes in the *C. acetobutylicum* asporogenous mutant M5 [21]. Finally, the ability to KD genes by antisense RNA has been successfully developed in *C. acetobutylicum* [22[•],23], and is now quite widely used in this and other clostridia including pathogenic strains. KD is especially suitable to examine phenotypes of genes whose KO or mutation may be lethal. Two reporter systems have been also developed [24,25]. Metabolic flux analysis was a well developed and most useful tool [26] and has been recently upgraded as a capability with the development of a genome-scale model [27^{••},28]. In fact, *C. acetobutylicum* is the organism where the original concept of flux analysis

was developed some 25 years ago [29]. Design of microarrays and the analysis protocols (both laboratory and computational) required adaptation of protocols from other organisms owing to the high A + T content of clostridia and the profound instability of the RNA but are now well developed ([30^{••},31[•],32,33[•]]). Standard proteomic tools have also been used with these organisms (e.g. [34]).

How to fix the major generic issues: aerointolerance, low cell densities, and limited sustainable viability

The major advantage of butyric acid and related clostridia is their powerful central primary metabolism (Figure 1). Specific carbon fluxes are very good (e.g. [13,14]), but cell densities are relatively low (around a max of 10–11 of absorbance at 600 nm (A_{600})), and the ability to sustain

Figure 1



Primary metabolism of *C. acetobutylicum*. Enzymes are abbreviated as follows, and gene names are italicized: phosphotransacetylase (PTA, *pta*); acetate kinase (AK, *ack*); thiolase (THL, *thl*); β -hydroxybutyryl dehydrogenase (BHBD, *bhbd*); crotonase (CRO, *cro*); butyryl-CoA dehydrogenase (BCD, *bcd*); CoA Transferase (CoAT, *ctfA*, *B*); acetoacetate decarboxylase (AADC, *adc*); butyrate kinase (BK, *buk*); phosphotransbutyrylase (PTB, *ptb*); alcohol/aldehyde dehydrogenase (AAD, *aad*); hydrogenase (HYDA, *hydA*); pyruvate decarboxylase (PDC, *pcd*). Note: AAD is believed to be the primary enzyme for butanol and ethanol formation, but additional genes that code for alcohol forming enzymes (*adhe2*, *bhA*, *bhB*, CAC3292, CAP0059) also exist. The involvement of PDC in ethanol formation has not been explored in these organisms, but given its good expression levels from microarray studies [52^{••}], a role can be assumed.

them viable over prolonged time periods is limited. Significantly, while anaerobiosis is essential for their powerful primary metabolism, their typically low aerotolerance complicates bioprocessing.

The issue of aerotolerance has been recently attended to [35^{••}]. It was shown that deletion of a peroxide repressor (PerR)-homologous protein in *C. acetobutylicum* resulted in prolonged aerotolerance, limited growth under aerobic conditions, higher resistance to H₂O₂, and rapid consumption of oxygen. Several genes were identified as putative members of the clostridial PerR regulon, including the heat shock protein Hsp21, a multifunctional reverse rubrerythrin that is proposed to play a crucial role in oxidative-stress defense. This work generated new opportunities to further enhance clostridial aerotolerance. The ability to improve the aerotolerance of clostridia will be most profound in the extremely sensitive to oxygen cellulolytic clostridia (*C. saccharolyticum*, *C. thermocellum*, etc.).

Low cell densities could derive from either butyric acid and/or acetic acid inhibition or some unknown quorum-sensing mechanism. The former could be potentially addressed by generic or specific mechanisms of acid tolerance, but none have yet been investigated. Quorum sensing mechanisms remain unexplored in these clostridia, but have been studied in pathogenic clostridia [36,37]. Some quorum sensing genes can be identified on the sequenced genomes of the solventogenic clostridia, and so there is a good likelihood that ME of quorum sensing might aid in the development of engineered strains that can achieve higher cell densities. The issue of cell density may be also related to the development of better growth media (the currently used media have never anticipated high cell densities from clostridia fermentations) as well as more suitable bioreactor operation, namely the use of fed-batch fermentations. Viability is a more difficult issue to address and is probably associated with their commitment to sporulation. It is an issue that deserves a systematic exploration.

How to make them grow on complex cellulosic substrates: the cellulosome and how to fix it

Cellulolytic clostridia degrade cellulose via the cellulosome [38]. This enzymatic complex is generally bound to the cell surface, contains motifs that bind to insoluble cellulose, and is made up of various cellulases that cleave oligosaccharides from insoluble cellulose. There are several sequenced organisms that contain complete and functional cellulosomes (coded by 11 to ca. 26 genes), and of notable interest are cellulolytic clostridia (incl. *C. phytofermentans*, *C. thermocellum*, and *C. cellulolyticum*), which produce acetate, lactate, and ethanol as primary metabolites. It has been also documented that butyric acid clostridia (none sequenced or well characterized) can effectively utilize cellulose without any genetic modifi-

cations [7^{••},39]. *C. acetobutylicum* contains an apparently complete cellulosome (a set of at least 11 genes/proteins, including dockerin domains; see below) [40] although it is not able to grow on cellulose effectively [41]. The reason for the inability to do so could derive from either gene silencing of an essential and function-limiting protein or an essential mutation in such a protein, or some inability to transport and assemble the cellulosome in the right cellular locale. This is a subject of active research by several groups.

Recent ME efforts suggest that this organism can be metabolically engineered to acquire a complete, functional, and efficient cellulosome that can in principle couple cellulose degradation to solventogenesis. A mini-CipA (the *cipA* gene encodes the *C. acetobutylicum* scaffolding protein CipA) polypeptide consisting of a family 3a cellulose-binding domain and two cohesin domains was overexpressed in *C. acetobutylicum*, yielding the *in vivo* formation of a minicellulosome [42]. Although not functional toward cellulose degradation, this study demonstrated the potential of ME for reconstituting recombinant cellulosomes. Another effort sought to create heterologous mini-cellulosomes in *C. acetobutylicum* with components from *C. cellulolyticum* and *C. thermocellum* [43,44^{••}]. The strategy was to create a mini-cellulosome containing different cellulases bound to a mini-scaffoldin. A first step toward this goal was to produce functional scaffoldins (miniCipC1, a truncated form of CipC from *C. cellulolyticum*, and the hybrid scaffoldin Scaf 3 from *C. thermocellum*). Both proteins were correctly expressed and were found to be functional. The next step was to correctly express and fold cellulases, and to determine if they bind to the scaffold. Thus, *man5K* encoding the mannanase Man5K from *C. cellulolyticum* was expressed alone or with *cipC1* (encoding miniCipC1) in *C. acetobutylicum*. Both recombinant strains secreted active Man5K but truncated when expressed alone. However, when *man5K* was coexpressed with *cipC1*, the recombinant strain secreted the full-length mannanase bound to miniCipC1 to generate a secreted, biologically functional heterocomplex. These studies suggest that *C. acetobutylicum* is a suitable platform host for the expression, assembly, and secretion of heterologous (mini) cellulosomes.

To undo the sporulation, and thus increase productivity and simplify bioprocessing: differentiation engineering?

The metabolism of *C. acetobutylicum* and of other solventogenic clostridia is biphasic in batch culture: first producing acetate and butyrate and later butanol, acetone, and ethanol. During growth, the production of acids lowers the pH of the culture, which combined with butyrate accumulation shifts the metabolism toward solvent production. Solvent formation is associated with re-uptake of the acids that are then converted into solvents (Figure 1). Solvent formation coincides with initiation of sporulation.

Upon consecutive vegetative transfers or in continuous culture cells frequently degenerate, that is, they become asporogenous and lose the capability to produce solvents. In *C. acetobutylicum*, degeneration derives from loss of the pSOL1 megaplasmid [45], which carries the key solvent formation genes, namely the *sol* operon (*aad-ctfA-ctfB*) (coding for the enzymes AAD, and CoAT, Figure 1) and the *adc* gene (coding for the enzyme AADC, Figure 1). Industrially, a solvent-producing, non-sporulating strain is most desirable because solventogenic clostridia produce solvents only during a rather narrow window of their sporulation program, probably during and/or the near stage of the characteristic, cigar-shaped, clostridial-cell form [1], which is a rather small fraction of the total cell population, and this limits the specific cell productivity. Furthermore, sporulating strains are not suitable for continuous or fed-batch fermentations owing to the commitment to sporulation and the likelihood of strain degeneration. Thus, the ability to undo the sporulation program without decreasing solvent formation would benefit specific cell productivity and simplify bioprocessing in that continuous or semi-continuous bioprocessing would improve process economics. Early efforts relied on random mutagenesis to identifying asporogenous, butanol producing clostridia as described in two patents [46,47]. Such mutants are not easy to characterize and are not readily amenable to further targeted genetic modifications. Significantly, they do not allow one to stop the sporulation process at a desirable sporulation stage that might be beneficial for the desirable bioprocess. Efforts to employ specific ME tools (KO and KD) for targeting specific sporulation stages have been more recently reported. A brief summary of clostridial sporulation is then in order.

Clostridial differentiation (i.e., sporulation) remains poorly understood. Sporulation has been well studied in *B. subtilis*. Sporulation mechanisms in *B. subtilis* and clostridia share some similarity, but many differences exist [2[•]]. A set of homologous sigma factors regulates differentiation in *B. subtilis* and also in clostridia. Sigma-factor expression occurs sequentially through sporulation, with specific sigma factors (SigH, SigF, SigE, SigG, SigK) present at different morphological stages [2[•]]. Sporulation in *B. subtilis* is initiated by a phosphorelay cascade, which responds to environmental and metabolic signals by phosphorylating Spo0A, the key transcription factor, which initiates sporulation. This phosphorelay system does not exist in clostridia [2[•]]. Expression of *spo0A* coincides with the onset of sporulation and other stationary phase phenomena in several clostridia [18,48,49]. Activated Spo0A induces the expression of key solventogenic genes in *C. acetobutylicum* [18,33[•]] (namely of the *sol* locus genes organized in two operons (*aad-ctfA-ctfB* and *adc*) located on the pSOL1 megaplasmid [45]) and in *C. beijerinckii* [50]. Among the genes upregulated by Spo0A ~ P are the *spoIIA* operon (which includes the

anti-anti sigma factor *spoIIAA*, the anti-sigma factor *spoIIAB*, and the early forespore specific gene *sigF*), *spoIIIE*, and the *spoIIIG* operon (which includes the gene for the mother cell-specific sigma factor *sigE*, and the forespore-specific sigma factor *sigG*). SpoIIIE is a phosphatase that activates (by dephosphorylation) SpoIIAA. Activated SpoIIAA interacts with SpoIIAB-SigF complexes, thus releasing SigF and allowing it to transcribe forespore-specific genes.

An attempt for decoupling solvent formation from sporulation was reported whereby asRNA KD was directed toward SpoIIIE [51[•]]. Downregulation of SpoIIIE delayed, but did not abolish, sporulation and had little if any impact on solvent (incl. butanol) titers [51[•]]. The expectation now is that other genes (such as *sigF*, *sigE* or *sigG*) may be more successfully targeted for inactivation to stop the sporulation program at a cellular stage where solvent production is the highest. An early evidence of this possibility was recently reported [52^{••}], whereby asRNA KD of two novel, apparently sporulation-related sigma factors (CAC1766 and CAP 0167) led to profound morphological changes, defective sporulation, and accompanied by improved metabolism and solvent formation.

A different approach is to start with asporogenous, non-solventogenic strains (such as strains M5 and DG1 of *C. acetobutylicum*, which have lost the pSOL1 megaplasmid [45]) and re-introduce or overexpress the desirable solvent formation genes. It was indeed shown that strains M5 and DG1 can be complemented by plasmids carrying the *aad* gene (Figure 1, expressed from its native promoter), and this leads to butanol formation, but at relatively low levels [45,53], while no acetone is produced because these strains do not carry the genes (Figure 1) necessary for acetone formation. A recent study [21] examined whether such strains can be made to produce higher levels of butanol similar to those of the WT strain. Such strains would be very desirable for an additional important reason: they would not produce acetone (a less desirable product), thus significantly increasing the selectivity of the process for butanol, and thus its economic appeal. Using the degenerate strain M5, the *aad* gene was overexpressed from the *ptb* promoter (Figure 1) to bypass the native regulation. This restored butanol production to WT 824 levels, but inability of the strain to produce acetone and thus re-assimilate the acids led to the production of high levels of acetate. To counter the large acetate production, thiolase (*thl*, Figure 1) overexpression, and inactivation of the acetate kinase (*ack*) genes were combined with *aad* overexpression. Both strains reduced acetate production, but butanol production was also decreased. Efforts to KO the butyrate kinase (*buk*) gene in the M5 strain failed, thus suggesting an important role for other pSOL1 genes in view of the fact that *buk* can be effectively knocked out in the WT 824 strain [17].

The generic and profound issue of metabolite (in)tolerance

Product tolerance is one of the most profound and most widely discussed and researched issues affecting the application of microbes for production of chemicals and fuels. In particular, a major concern in biofuel production (as well as in bioremediation applications and whole-cell biocatalysis in various organic media) is the inhibitory effects of alcohols and other solvents, but carboxylic acid toxicity and the combined effect of the two are very serious concerns, as well. Research on the impact of alcohols and ketones on *E. coli* and other Gram-negative organisms has shown that intercalation of solvents within the lipid bilayer increases membrane fluidity and also affects lipid-protein interactions integral to membrane function [54]. There has been limited progress on the development of solvent (such as ethanol) tolerant strains. Much effort has been based on the concept that ethanol (and more broadly solvent) tolerance can be controlled by a single gene, or a few genes. It is particularly instructive to note, however, that the tolerant phenotype is the result of a several simultaneous mechanisms of action, including molecular pumps, changes in membrane properties, changes in cell wall composition, and altered energy metabolism, thus making implementation of tolerance strategies a difficult task.

Much less is known about the impact of solvents and toxic chemicals in general on Gram-positive organisms such as clostridia and bacilli [55^{*}]. As summarized [55^{*}], when clostridia are exposed to butanol, the ratio of saturated to unsaturated fatty acids incorporated in the membrane lipid bilayer increases, presumably to compensate for the fluidity increase imposed by the solvent. Solvents inhibit membrane-bound ATPases, resulting in a drop in internal pH and abolishment of the Δ pH gradient across the membrane. Butanol also inhibits glucose uptake, thus inhibiting energy generation that is compounded by an independent drop in intracellular ATP levels. Yet, improvements to solvent tolerance and titers in *C. acetobutylicum* have not come about from ME of membrane functions. Instead, over-expression of the Class I stress-response operon *groESL* resulted in increased solvent tolerance and solvent titers 33% higher than a plasmid-control strain [56^{*}], indicating a denaturing effect of solvents on functional enzymes and a role for stress proteins in solvent tolerance. A separate approach was based on the assumption that the dramatic changes associated with sporulation [2^{*}] would alter tolerance to butanol. Indeed, over-expression of the master regulator of sporulation Spo0A (strain 824(pMSPOA)) resulted in enhanced and accelerated sporulation, increased butanol tolerance and prolonged metabolism under butanol stress [57]. Microarray analysis of 824(pMSPOA) showed that most (but not all) of the differentially transcribed genes were general stress response genes.

More recently, a genomic library based on sheared genomic DNA was constructed and employed for the selection and identification of genes imparting solvent tolerance [55^{*}]. An enrichment protocol based on serial transfer into successively higher butanol concentrations resulted in the successful identification of solvent-tolerance gene fragments. Among the library inserts enriched through serial transfer were a set of transcriptional regulators (CAC0977, CAC1463, CAC1869, and CAC2495) with the potential to impact multiple tolerance mechanisms. The most enriched gene (CAC1869) was found to be homologous to XRE (xenobiotic responsive element) transcriptional regulators, which contribute to an array of tolerance mechanisms in other microorganisms. This gene was re-cloned to generate a recombinant *C. acetobutylicum* strain, which showed a 90% increase in butanol tolerance compared with the plasmid control strain [55^{*}]. These limited studies underscore the complexity of solvent toxicity and warrant a genomic approach for both discovering and employing solvent-tolerance genes.

Global regulatory metabolic engineering through protein and metabolite regulators: foretelling and anticipating the synthetic biology bonanza?

Compared with pathway engineering, the employment of regulatory proteins or proteins affecting a larger set of pathways, programs, or signal transduction leading to desirable phenotypes is likely to be as important and in some ways more profound in achieving the desirable phenotype. It was discussed already that GroESL over-expression imparts butanol tolerance [56^{*}]. The mechanism can be generically summarized as that of stabilizing proteins and cellular structures under stress thus making possible prolonged cellular metabolism and increased butanol formation. Yet, microarray analysis of strain 824(pGROE1) in the absence of butanol stress revealed increased expression of many metabolic and regulatory genes and decreased expression of the other major stress response genes, thus suggesting that GroESL functions as a modulator of the CIRCE stress regulon. A more complex regulon emerged upon exposure of 824(pGROE1) to butanol stress [58], again highlighting the important role of GroESL as a regulator of complex cellular programs. We also discussed the importance of the unknown transcriptional regulator coded by CAC1869 in imparting tolerance to butanol stress [55^{*}]. The mechanism is unknown, but the effect is quite profound and the ME strategy is simple and fast. Similarly, we also briefly discussed the impact of differentiation engineering of the sporulation program of *C. acetobutylicum* using asRNA downregulation of sporulation-specific sigma and related factors [51^{*},52^{**}].

Perhaps the earliest documented ME intervention in clostridia employing global engineering of the transcriptional machinery is that of the *C. acetobutylicum* CAP0161-

coded protein (the so-called SolR) [59]. Overexpression of SolR in strain ATCC 824(pCO1) suppressed solvent formation owing to the suppressed transcription of the *sol* locus genes. Inactivation of *solR* in *C. acetobutylicum* via homologous recombination yielded mutants that displayed increased solvent fluxes, earlier induction of solvent genes, and prolonged solvent production. The gene was then assessed as being a transcriptional repressor of the *sol* operon (thus the name SolR), but this was later challenged [60,61]. Nevertheless this *solR* KO strain and its derivative, which includes *aad* overexpression off a plasmid, produced the highest known levels of butanol and solvents (240 and 430 mM, respectively), thus suggesting an increased butanol tolerance also. This is clearly a regulatory mutant impacting the circuit that regulates solvent-gene expression and remains to be elucidated and further explored.

Finally, there exists the case of a regulatory small molecule (butyrate phosphate (BuP)) affecting the regulatory circuits of solvent formation. Green *et al.* [17] used a non-replicating vector strategy to KO the *buk* gene of the butyrate formation pathway from butyryl-CoA (Figure 1). Strain (PJC4BK) produced significantly reduced acids and very high levels of butanol, and slightly higher levels of solvents when combined with *aad* overexpression (226 mM butanol, 66 mM acetone and 98 mM ethanol) [14]. The impact of *buk* was verified by an asRNA down-regulation, but surprisingly, when the other gene (*ptb*, Figure 1) in the butyrate-formation pathway was down-regulated, the cells grew poorly and produced low levels of solvents but large levels of lactate. On the basis of these and related results, it was hypothesized [14,62] that the BuP molecule that is the intermediate between butyryl-CoA and butyrate (Figure 1) has an important regulatory role, whereby upon the *buk* KO or KD, Bu-P accumulates to higher levels and induces solvent-formation enzymes. When intracellular levels of BuP were measured in the PJC4BK and several control strains (WT, M5, and a *pta* mutant (Figure 1)), this hypothesis was found to be correct, with the PJC4BK strain showing the highest BuP levels [63]. The data from this study suggest that BuP, but not acetyl-P, is a regulatory molecule that may act as a phosphodonor of transcriptional factors. Microarray transcriptional analysis demonstrated that high BuP levels corresponded to upregulation of solvent formation and stress genes [63].

Biobutanol production: what could we expect from ME?

For butanol production, process economics would be greatly improved (i) with continuous or repeated fed-batch fermentations even without any other strain improvements; (ii) improved butanol tolerance so that higher titers can be achieved [butanol titers rarely exceed 12–13 g/l in unmodified strains, and economic analyses show that if the final butanol titers were raised from 12 to

19 g/l, the separation costs would be cut in half, as already discussed]; and (iii) improved butanol selectivities (higher fraction of butanol in the final product). Regarding the implementation of a continuous process, we already discussed the need for and ME efforts to develop strains that do not sporulate or degenerate but produce high levels of solvents. We also discussed the need for and efforts to achieve higher butanol tolerance. Regarding increased butanol selectivities, efforts have been made to ME the organism to reduce or eliminate acetone production. In the WT genetic background, asRNA-based downregulation of CoA transferase (CoAT, the first enzyme in the acetone-formation pathway, Figure 1) combined with *aad* overexpression from its native promoter, resulted in increased butanol to acetone selectivity, but overall reduced butanol yields and titers [23]. Recently, *aad* overexpression using the phosphotranbutyrylase (*ptb*) promoter combined with CoAT downregulation led to early production of high alcohol (butanol plus ethanol) titers and overall solvent titers of 30 g/l [64]. Metabolic flux analysis revealed the likely depletion of butyryl-CoA, and further efforts to reduce ethanol and acetate production did not yet prove successful [64], but strategies were suggested for achieving that goal.

Future needs and directions

It should be clear now that better genetic tools are necessary for carrying out complex ME tasks quickly and effectively. Genomic integration is a key tool. While recent work has made possible to KO a gene easier, integrating genes into the chromosome and removing the antibiotic selection markers for further genetic manipulations, let alone quickly and efficiently as in *E. coli* and yeast cells, remains a grand challenge that must be addressed. The need for inducible and tunable promoters, more selection markers, and fluorescence probes/proteins in order to follow genetic alterations also remain high on the list of desirables. Development of methods for efficient cell transformation using large plasmids let alone fosmids or cosmids, in order to express large sets of genes, are also of high priority. Auxotroph mutants for genetic work are also urgently needed. Superior, high-throughput genomic tools can be now expected and in some way taken for granted, in view of the fact that such tools are largely organism independent, and will thus require but a minor adaptation to these organisms.

Development of solventogenic clostridia that quickly and effectively utilize cellulose in a deregulated way would enormously simplify bioprocessing. Xylan, a polymer whose main component is xylose, is the most abundant renewable polysaccharide in plant tissues after cellulose. Unlike cellulose, whose composition of linear 1,4- β -linked polyglucose chains is consistent from plant to plant, xylan has a variable composition, which ranges from linear 1,4- β -linked polyxylose chains to highly branched heteropolysaccharides depending on the plant.

Xylan degrading cellulolytic strains appear to use a modified cellulosome to attack xylan, and while some butyric acid clostridia are capable of effective xylan utilization, this trait is highly variable necessitating additional development, including ME to generate effective industrial strains for simultaneous cellulose and xylan degradation. In general, deregulating the use of various simpler sugars (hexoses, pentoses and disaccharides) so that they can be used simultaneously is an important goal for effectively feeding the fermentation with biomass hydrolyzates.

Work done so far should anticipate the development of ME strains that have high butanol yields and selectivities, and strains that do not sporulate or degenerate, that tolerate higher oxygen levels, can grow to much higher densities, and thus suitable for continuous, high-density, simple bioprocessing. The development of these strains will require significant effort and resources, but the probability of success is high.

On the solvent tolerance front, the probability of success is difficult to assess, but there is a good likelihood that reprogramming the cellular machinery of membrane and cell wall biosynthesis may result in dramatic tolerance improvements. Furthermore, the capability of combining several single-gene improvements into a single strain may also result in large tolerance improvements. Finally, isolation and sequence of more targeted strains may expand the genetic 'space' of the tolerance phenotype and offer new ideas and opportunities.

Conflict of interest

The author declares there is no conflict of interest.

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