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MINI-REVIEW

Application of new metabolic engineering tools for *Clostridium acetobutylicum*

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Abstract The renewed interests in clostridial acetone-butanol-ethanol (ABE) fermentation as a next-generation biofuel source led to significantly intensified research in the past few years. This mini-review focuses on the current status of metabolic engineering techniques available for the model organism of ABE fermentation, *Clostridium acetobutylicum*. A comprehensive survey of various application examples covers two general issues related to both basic and applied research questions: (i) how to improve biofuel production and (ii) what information can be deduced from respective genotype/phenotype manipulations. Recently developed strategies to engineer *C. acetobutylicum* are summarized including the current portfolio of altered gene expression methodologies, as well as systematic (rational) and explorative (combinatorial) metabolic engineering approaches.

Keywords Biofuel · Butanol · Clostridia · Knockout · Mutagenesis · Screening

Introduction

General interests in microbial fermentation processes for biofuels as a promising alternative to petrochemical production are steadily increasing, particularly in terms of technology development and economic competitiveness (Chu and Majumdar 2012; Caspeta et al. 2013). One of such advanced biofuels is 1-butanol, a C_4 primary alcohol which is also an important commodity chemical used as solvent or precursor for diverse industrial compounds such as butyl acrylate and methacrylate esters (Mascal 2012). The usage of butanol as a fuel or fuel additive has several advantages over ethanol, notably a higher energy density, a lower volatility, and it is less hygroscopic and less corrosive. Therefore, butanol is entirely compatible with the existing infrastructure for liquid fuel transportation and storage, and current gasoline engines do not require any technical modifications (Jin et al. 2011). Besides chemical synthesis, butanol can be obtained biologically, and thus far, microorganisms belonging to the genus *Clostridium* are the only known organisms capable to naturally synthesize 1-butanol (Papoutsakis 2008; Lee et al. 2008; Jang et al. 2012a).

Already in 1862, this unique fermentative metabolism was described by Louis Pasteur who stated "l'alcool butylique est un produit ordinaire de la fermentation butyrique" (Pasteur 1862). Fifty years later, Chaim Weizmann discovered strain BY, which was later classified as *Clostridium acetobutylicum*, as a biocatalyst for the conversion of starch into butanol and acetone. The latter solvent was of particular importance, because acetone was needed in vast quantities for the production of cordite during the First World War. Thus, this production process called acetone-butanol-ethanol (ABE) fermentation quickly grew to the commercial scale. After the war, ABE fermentation was a valuable source of butanol to satisfy the increasing demand for this solvent, e.g. in the fast-growing automobile industry in the 1920s. The ammunition-dependent need for acetone re-emerged during the Second World War, and eventually, the ABE fermentation became one of the largest industrial bioprocesses (Jones and Woods 1986). Most of the ABE production plants in Western countries were closed in the 1950s and early 1960s due to cheaper petrochemical production routes of the solvents (Jones and Woods 1986; Kumar and Gayen 2011).

However, ABE fermentation regained much interests in academia and industry in recent years, and several either retrofitted or new industrial plants are currently under construction or even operating in the People's Republic of China

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(Ni and Sun 2009; Dong et al. 2012a). Fundamental technical improvements in bioprocess optimization such as product recovery and utilization of appropriate feedstocks have been reviewed recently (e.g. Gu et al. 2011; Kumar and Gayen 2011; Jang et al. 2012c; Jurgens et al. 2012; Mariano and Filho 2012; Moholkar et al. 2012; Ranjan and Moholkar 2012; Bankar et al. 2013; Xue et al. 2014).

Metabolic engineering of microorganisms for biofuel production does not only comprise a powerful portfolio for sustainable strain improvement but can also provide beneficial insights into cellular functioning. Basically, two general strategies for biobutanol production can be and have been pursued: (i) implementation of heterologous butanol biosynthesis in a suitable host organism and (ii) enhancement of natural butanol producers (Alper and Stephanopoulos 2009; Gronenberg et al. 2013). The present mini-review will focus exclusively on the latter issue, and recent advances in developing and employing genetic tools to engineer *C. acetobutylicum* as the model organism of ABE fermentation will be summarized.

Physiology of *C. acetobutylicum*

Natural ABE fermentation is exclusively performed by solventogenic clostridia, a group of strictly anaerobic, endospore-forming, Gram-positive bacteria which ubiquitously occur in soil, sludge and wastewater (Berezina et al. 2012). In general, the typical life cycle is tightly associated with two metabolic phases designated as acidogenesis and solventogenesis. During exponential growth, *C. acetobutylicum* metabolizes carbohydrates to acetic and butyric acids while generating a surplus of adenosine triphosphate (ATP) in addition to the glycolytic ATP in order to produce as much energy as possible under anaerobic conditions (Fig. 1). This extensive glycolytic flux and acid production is accompanied by a severe acidification of both the environment and the cell itself, most likely resulting in a primary stress signal for counteracting the rapid pH decrease. In addition, the non-respiratory metabolism unconditionally requires a balance between formation and utilization of reducing equivalents that necessitates adjustments of the primary metabolism (Jones and Woods 1986; Lütke-Eversloh and Bahl 2011). This is commonly represented by the reduced/oxidized nicotinamide adenine dinucleotide (phosphate) ratio NAD(P)H/NAD(P)$^+$ as a typical cell signal and an important parameter among others to alter the metabolism (Grupe and Gottschalk 1992; Wietzke and Bahl 2012). This metabolic shift occurs during the transition from exponential to stationary growth and initiates solventogenesis. The previously formed acids are re-assimilated, and the solvents acetone, butanol and ethanol are formed, usually at a ratio of 3:6:1 (Fig. 1). The metabolic phases are accompanied by different morphological states of the life cycle. While exponentially

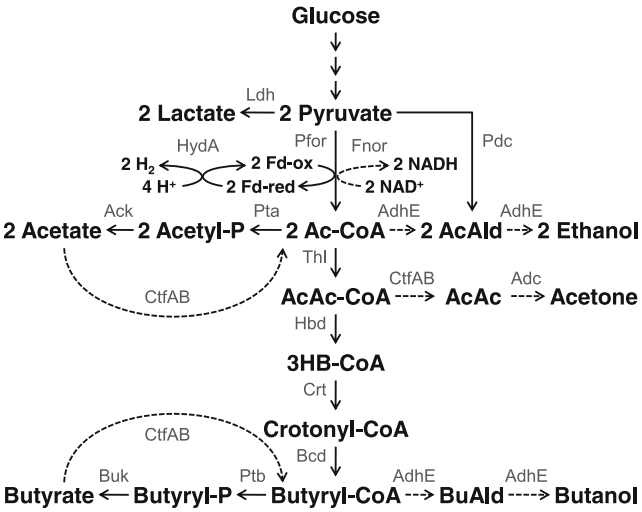

Fig. 1 Central metabolism of *C. acetobutylicum*. Enzymes: Ldh, lactate dehydrogenase; HydA, hydrogenase; Pfor, pyruvate:ferredoxin oxidoreductase; Fnor, ferredoxin:NAD(P) + xidoreductase; Pdc, pyruvate decarboxylase; Pta, phosphotransacetylase; Ack, acetate kinase; AdhE, aldehyde/alcohol dehyrogenase; CtfAB, acetoacetyl-CoA:acyl-CoA transferase; Adc, acetoacetate decarboxylase; Thl, thiolase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase, Bcd, butyryl-CoA dehydrogenase complex; Ptb, phosphotransbutyrylase, Buk, butyrate kinase. Metabolites: Acetyl-P, acetylphosphate; Ac-CoA, acetyl-CoA; AcAld, acetaldehyde; AcAc-CoA, acetoacetyl-CoA; AcAc, acetoacetate; 3HB-CoA, 3-hydroxybutyryl-CoA; Butyryl-P, butyrylphosphate; BuAld, butyraldehyde. The *dashed lines* indicate solventogenic reactions

growing cells are typically rod-shaped, stationary phase cells become a swollen, cigar-shaped form due to the accumulation of granulose, a starch-like storage polysaccharide which serves as carbon and energy source for the subsequent sporulation process. After maturation, the spores are released from the mother cell and can survive for many years until favourable environmental conditions promote germination (Dürre and Hollergschwandner 2004).

Targeted manipulation of gene expression

Plasmids for gene overexpression

The first recombinant strains of *C. acetobutylicum* for homologous gene overexpression were described in 1992, although construction of suitable cloning vectors and transformation via conjugation or electroporation were reported a few years earlier (Oultram et al. 1988; Truffaut et al. 1989; Young et al. 1989; Willims et al. 1990). Based on a *Bacillus subtilis*/*C. acetobutylicum* shuttle vector harbouring the replicon derived from pIM13 of *B. subtilis* and a macrolide lincosamin resistance gene as selection marker, acetone and butyrate biosynthetic genes were homologously overexpressed (Lee et al. 1992b; Mermelstein et al. 1992, 1993). The main obstacle resulting in low transformation efficiencies and plasmid

instability is the native, highly active restriction endonuclease *Cac*824I of *C. acetobutylicum*, and multiple recognition sites frequently occur in standard *Escherichia coli* cloning vectors. Since a methyltransferase for the respective recognition site (5′-GCNGC-3′) was commercially not available, a suitable in vivo methylation protocol was developed which is still commonly used nowadays. Plasmids pAN1 and pAN2, equipped with compatible *E. coli* replicons and antibiotic resistance genes, express the ϕ3TI methyltransferase gene of the *B. subtilis* phage ϕ3TI and protects plasmids to be transformed into *C. acetobutylicum* from hydrolysis (Mermelstein and Papoutsakis 1993; Heap et al. 2007). An alternative strategy to prevent *Cac*824I restriction of recombinant plasmids is the employment of *Cac*824I-deficient host strains which allows omission of the additional transformation for in vivo methylation in *E. coli* and subsequent purification of methylated plasmids prior to electroporation into *C. acetobutylicum* (Soucaille et al. 2008; Dong et al. 2010).

Due to the limitation of functional antibiotic markers, the number of shuttle vectors established for the use in *C. acetobutylicum* is quite small, and most overexpression studies were based on derivatives of the pSOS94 and pIMP1 plasmids (Lee et al. 1992a; Mermelstein et al. 1992; Tummala et al. 1999). Hence, N. P. Minton and coworkers developed the pMTL80000 plasmid series, a standardized modular *E. coli*/*Clostridium* shuttle vector system which can be adapted as required. It consists of four basic modules comprising different replicons, markers and additional features such as multiple cloning sites or the *tra* genes for conjugative transfer, which can be assembled in any combination due to the common restriction sites (Heap et al. 2009).

In order to promote plasmid-borne gene expression, native clostridial promoters like the *thl*, *ptb* and *adc* promoters have widely been used, and their expression profiles are well known (Tummala et al. 1999; Feustel et al. 2004). Depending on the type of overexpression, inducible gene expression systems rather than constitutive promoters can be useful is some cases. However, the number of inducible promoter/repressor systems developed for *C. acetobutylicum* is still limited. Girbal et al. used the *xylA* promoter and *xylR* repressor from *Staphylococcus xylosus* to generate a xylose-inducible expression vector for *C. acetobutylicum* (Girbal et al. 2003). The renowned *lac* expression system was fused with the *fdx* promoter from *Clostridium pasteurianum*, yielding the artificial *lacI*-repressed *fac* promoter, which can be induced in *C. acetobutylicum* by addition of 1 mM IPTG (Heap et al. 2007). More recently, the portfolio of inducible promoters was complemented by the lactose-inducible *bgaL* promoter and the anhydrotetracycline-inducible Pcm-2tetO1 promoter, allowing reliable stringency and a broad range of inducibility (Al-Hinai et al. 2012; Dong et al. 2012b).

Chromosomal integration for gene overexpression

With respect to industrial applications of suitable production strains, plasmid-based gene expression is less desirable because of the need for plasmid maintenance involving, for example, addition of expensive antibiotics. Therefore, stable integration of homologous or heterologous genes under the control of a useful promoter is a preferred strategy for the design of potential producers. As described below, chromosomal manipulation of solventogenic clostridia is particularly difficult, and notable breakthroughs in this field were achieved only recently. However, the techniques explained in the last paragraph of this section allow both targeted gene knockout and integration of large DNA fragments of up to 6.5 kbp into the chromosome (Al-Hinai et al. 2012; Heap et al. 2012). Employing this cargo technique, *C. acetobutylicum* was engineered for isopropanol production by heterologous expression of the secondary alcohol dehydrogenase gene from *Clostridium beijerinckii* from the chromosome, resulting in the conversion of acetone into isopropanol (Heap et al. 2012). More recently, this method was successfully used to generate a recombinant *C. acetobutylicum* strain which secreted various synthetic cellulosome components, paving the way for future consolidated bioprocessing strategies (Kovács et al. 2013).

Integrative plasmids for gene inactivation

Already in 1996, the first knockout mutants of *C. acetobutylicum* with defects in the central metabolic pathways were reported, i.e. mutants of the aldehyde/alcohol dehydrogenase (*adhE1*), phosphotransacetylase (*pta*) and butyrate kinase (*buk*) genes (Green and Bennett 1996; Green et al. 1996). Non-replicative suicide plasmids were used for homologous recombination into the respective genes, but only single-crossover events by a Campbell-like integration of the entire plasmids were obtained. The same strategy was employed to inactivate SolR, a putative regulator of solventogenesis located upstream of the *sol* locus (Nair et al. 1999). Although the mutant exhibited an improved solventogenic phenotype, the function of SolR as a transcriptional repressor was critically discussed by others (Thormann and Dürre 2001; Thormann et al. 2002). The use of replicating plasmids was also reported for successful integration into a specific gene as demonstrated by inactivation of the *spo0A* gene encoding the master regulator of sporulation in *C. acetobutylicum* (Harris et al. 2002). This approach was recently combined with the expression of a recombinant resolvase gene (*recU*) from *B. subtilis* in order to delete the sigma factor genes *sigE* and *sigG* (Tracy et al. 2011). However, the obtained mutants also comprised only single-crossover events, indicating that heterologous RecU did not promote the Holliday junction resolution during the recombination event.

Antisense RNA for gene downregulation

Gene expression can be non-covalently modulated by in vivo hybridization of complementary messenger RNA (mRNA) transcripts, so-called antisense (as) RNA, which specifically inhibits gene translation (Thomason and Storz 2010). Employing plasmid-encoded asRNA against the butyrate biosynthetic genes *ptb* and *buk*, this strategy was shown to successfully reduce respective enzyme activities. However, the butyrate production by the recombinant strains was not reduced (Desai and Papoutsakis 1999). In contrast, targeting the acetone biosynthetic pathway, asRNA constructs against the *ctfB* gene encoding the acetoacetyl-CoA:acyl-CoA transferase subunit B in fact reduced acetone production in *C. acetobutylicum*, whereas the asRNA against the acetoacetate decarboxylase gene *adc* was not effective (Tummala et al. 2003b). Combination of *ctfA* downregulation and *adhE1* overexpression did not only restore butanol formation; the engineered strain exhibited a drastically increased ethanol production (Tummala et al. 2003a; Sillers et al. 2009). Lastly, asRNA was used to investigate the role of the sporulation regulator SpoIIE, and downregulation of *spoIIE* resulted in prolonged and elevated solventogenesis as well as in a delayed initiation of endospore formation (Scotcher and Bennett 2005).

ClosTron and related techniques for gene inactivation

Since genome manipulations by homologous recombination were particularly difficult in *C. acetobutylicum* and other clostridia, implementation of mobile group II introns for targeted gene inactivation provided a novel useful tool, probably an overdue breakthrough for clostridial genetics (Kuehne and Minton 2012). Group II introns are catalytically active RNAs comprising a multi-domain intron-encoded protein which mediates sequence recognition and self-splicing. Thus, alteration of only a few bases in the recognition site allows individual re-targeting for virtually any chromosomal gene (Karberg et al. 2001). The TargeTron system, which is commercially available from Sigma-Aldrich, employs this technique for insertional mutagenesis and is based on the *L1.LtrB* intron from *Lactococcus lactis*. It has been further engineered by including a retrotransposition-activated marker providing erythromycin resistance after intron insertion, and the functionality has been demonstrated in various clostridial species, including solventogenic strains (Heap et al. 2007; Wang et al. 2013). This so-called ClosTron system was further optimized, most notably by incorporation of flippase recognition target (FRT) sites flanking the marker cassette to allow flippase-mediated excision and thus multiple rounds of intron-mediated mutagenesis (Heap et al. 2010).

Interestingly, the *L1.LtrB*-intron-based mutagenesis does not necessarily require selective pressure by an antibiotic marker, because intron insertions occur frequently and were shown to be stable and reproducible (Shao et al. 2007; Jiang et al. 2009). Avoiding chromosomal marker excision and plasmid curing also facilitates the procedure of generating multiple-knockout mutants, as demonstrated by Jang et al. (2012b). Alternating use of erythromycin and thiamphenicol resistance genes for the mutagenesis plasmids harbouring the re-targeted group II introns, various gene knockout mutants including three double- and two triple-knockout strains of *C. acetobutylicum* were generated (Jang et al. 2012b).

Another variant for gene inactivation in *C. acetobutylicum* which combined both group II intron and homologous recombination techniques was described by Jia et al. (2011). The first step includes the targeted integration of the intron into the respective gene and selection of erythromycin-resistant clones. In the second mutagenesis step, multiple subculturing in the absence of erythromycin is required to identify those mutants in which the intron was deleted by homologous recombination. However, this alternative gene deletion method is very elaborate and time consuming because of successive transfers and large numbers of colonies to be screened; e.g. 648 clones of a chromosomal knockout and 1,998 clones of a pSOL1 knockout mutant had to be analysed to identify positive clones (Jia et al. 2011).

Allelic exchange mutagenesis

Although the ClosTron and related knockout strategies were shown to successfully inactivate a variety of genes in *C. acetobutylicum*, insertional mutagenesis may also cause polar effects of the integrated group II intron, and intrinsic instability of a ClosTron mutant has also been reported (Steiner et al. 2011). However, double-crossover mutants could be obtained only recently after establishment of a suitable selection system for the second crossover event. Allele-coupled exchange (ACE) was developed to either knockout genes or integrate large DNA fragments into the genome (Heap et al. 2012). Instead of using a plasmid-encoded counterselection marker, the method relies on a selectable phenotype based on the well-known uracil auxotrophy plus antimetabolite toxicity principle: First, uracil-auxotrophic mutants were selected which require uracil supplementation for growth. Second, addition of 5-fluoroorotic acid prevents growth of those mutants with a functional *pyrE/pyrF* gene, because they are sensitive towards the toxic antimetabolite which results from 5-fluoroorotic acid cleavage, and thus, only double-crossover mutants without integrated plasmid are selected (Heap et al. 2012). An alternative approach to generate stable deletion mutants of *C. acetobutylicum* was also published recently by E. T. Papoutsakis and coworkers. For the selection of double-crossover mutants, the *E. coli mazF* toxin gene, coding for an mRNA interferase, was coupled to a lactose-inducible promoter, enabling

counterselection in the presence of lactose (Al-Hinai et al. 2012). Furthermore, FRT sites were included adjacent to the thiamphenicol resistance cassette for subsequent excision of the marker and thus allowing 'clean' deletions and marker recycling. In order to facilitate plasmid curing, a plasmid carrying an asRNA against the *repL* replicon fused to the same lactose-inducible promoter was also constructed (Al-Hinai et al. 2012).

Systematic metabolic engineering

Engineering butanol tolerance

Butanol toxicity constitutes an important limiting factor of industrial ABE fermentation, because product tolerance is usually below 20 g/l (Liu and Qureshi 2010). The major stress responses of the cells include changes in the membrane composition to counteract the fluidity increase as well as drastically increased expression of heat shock protein (HSP) encoding genes (Tomas et al. 2004; Ezeji et al. 2010; Nicolaou et al. 2010; Janssen et al. 2012). Therefore, the HSP genes *groESL* were homologously overexpressed in *C. acetobutylicum*, leading to significantly improved robustness and solvent titres, and continuative transcriptome analyses indicated that other HSPs might have a similar effect (Tomas et al. 2003, 2004; Alsaker et al. 2010). Overexpression of the HSP genes *grpE* and *htpG* did effectively improve butanol tolerance traits, but higher solvent production could not be achieved, indicating that butanol tolerance and production capabilities of *C. acetobutylicum* are not necessarily linked together (Mann et al. 2012). Similarly, increased expression levels of the cyclopropane fatty acid synthase gene *cfa* impacted the cell membrane composition and thus exerted a positive effect on acid and solvent tolerance, but the recombinant *C. acetobutylicum* strain was significantly impaired in solvent production (Zhao et al. 2003).

As a new target for systematic engineering of butanol tolerance, the *gshAB* genes from *E. coli*, coding for γ-glutamate-cysteine ligase and glutathione synthetase, were overexpressed in *C. acetobutylicum*, resulting in a much more robust phenotype in terms of butanol resistance and aerotolerance (Zhu et al. 2011). This strategy was subsequently extended by Hou et al.: while disrupting the *adc* gene by integration of the *gshAB* cassette, both glutathione-mediated robustness and reduced acetone formation, an unwanted by-product (see "Engineering reduced by-products" section), were achieved. Further, systematic engineering by overexpression of genes involved in solventogenesis led to high-butanol titres of up to 15 g/l, an almost threefold increase as compared to the wild-type strain *C. acetobutylicum* ATCC 824 under similar cultivation conditions (Hou et al. 2013).

Interestingly, a negative control factor for butanol resistance was also described: Based on similarities to eukaryotic alcohol-binding regulators, two adjacent genes of unknown functions were identified in a comparative proteomic approach. Inactivation of either or both genes in *C. acetobutylicum* DSM 1731 clearly lowered the sensitivity towards butanol stress, whereas homologous overexpression of both genes inhibited growth in the presence of 1 vol% butanol (Jia et al. 2012).

Engineering reduced by-products

The fermentative metabolism of *C. acetobutylicum* and related solventogenic clostridia typically comprises a mixture of end products at varying ratios (Fig. 1). As a classical biotechnological principle of strain optimization, the formation of by-products must be reduced or eliminated to achieve higher yields of the desired product. Therefore, first metabolic engineering attempts implemented this strategy as soon as respective genetic tools were developed. Employing integrative plasmids for gene knockout, key enzymes of the acetate and butyrate biosynthetic pathways were targeted in order to increase the carbon flux towards butanol (Green et al. 1996). A few years later, asRNA constructs were generated to down-regulate enzymes of the butyrate and acetone pathways (Desai et al. 1999; Desai and Papoutsakis 1999; Tummala et al. 2003b). However, the vast majority of defined knockout mutants with inactivated enzymes of the central carbon metabolism were generated only recently according to the late development of new gene knockout methodologies. Table 1 shows a survey of all knockout mutants of *C. acetobutylicum* targeted for altered acid or solvent pathways published thus far, including the major phenotypic characteristics of the mutants.

Interestingly, knockout of either Pta or Ack of the acetate biosynthetic pathway did not result in clear acetate-negative phenotypes, which was similarly true for Buk of the analogous butyrate pathway, suggesting some enzymatic complementation of the corresponding C_2- and C_4-acidogenic pathways (Table 1 and Fig. 1). With respect to butyrate synthesis, only three studies reported a distinct butyrate-negative phenotype of *C. acetobutylicum* mutants, comprising an intron-mediated disruption of the *hbd* or *ptb* gene, respectively (Lehmann and Lütke-Eversloh 2011; Lehmann et al. 2012b; Cooksley et al. 2012). All single-knockout mutants with disrupted acetone biosynthetic enzymes exhibited reduced or zero, respectively, acetone production with concomitantly elevated acetate titres, indicating a strong relation between acetate re-assimilation and acetone formation (Table 1). Therefore, disruption of Pta was introduced into these acetone-impaired mutants, and the novel double-knockout mutants of *C. acetobutylicum* exhibited both low-acetate and low-acetone levels. However, instead of an improved butanol production, these mutant strains

Table 1 Knockout mutants of *C. acetobutylicum* with defects in the central fermentative metabolism

Mutant strain of *C. acetobutylicum*	Inactivated protein	Pathway	Product pattern						Reference
			Acetate	Butyrate	Lactate	Acetone	Ethanol	Butanol	
PJC4PTA[a,d]	Phosphotransacetylase (Pta)	Acetate biosynthesis	−	+	uk	o	o	o	Green et al. 1996
pta::int(80)[b]	Pta	Acetate biosynthesis	o	o	o	o	o	o	Lehmann et al. 2012a
EKW[b]	Pta	Acetate biosynthesis	−	o	uk	+	+	+	Jang et al. 2012b
WUR AK[c]	Acetate kinase (Ack)	Acetate biosynthesis	−	o	o	o	o	o	Kuit et al. 2012
Cac-*ack*349a::CT[b]	Ack	Acetate biosynthesis	−	o	+	o	+	+	Cooksley et al. 2012
AKW[b]	Ack	Acetate biosynthesis	−	+	uk	o	o	o	Jang et al. 2012b
PJC4BK[a,d]	Butyrate kinase (Buk)	Butyrate biosynthesis	+	−	uk	−	+	+	Green et al. 1996
824 (buk^{-})[b]	Buk	Butyrate biosynthesis	uk	uk	uk	o	+	+	Shao et al. 2007
BKW[b]	Buk	Butyrate biosynthesis	−	−	uk	+	+	+	Jang et al. 2012b
ptb::int(87)[c]	Phosphotransbutyrylase (Ptb)	Butyrate biosynthesis	+	- -	o	o	++	−	Lehmann et al. 2012b
Cac-*ptb*505a::CT[b]	Ptb	Butyrate biosynthesis	++	- -	++	- -	++	−	Cooksley et al. 2012
PKW[b]	Ptb	Butyrate biosynthesis	o	o	uk	o	o	+	Jang et al. 2012b
BEKW[b]	Pta and Buk	Acetate and butyrate biosynthesis	−	o	uk	−	o	+	Jang et al. 2012b
BBEKW[b]	Pta, Buk and Buk II	Acetate and butyrate biosynthesis	−	o	uk	−	o	−	Jang et al. 2012b
PEKW[b]	Pta and Ptb	Acetate and butyrate biosynthesis	−	+	uk	−	o	−	Jang et al. 2012b
2018adc[b]	Acetoacetate decarboxylase (Adc)	Acetone biosynthesis	++	o	uk	−	−	−	Jiang et al. 2009
adc::int(180)[b]	Adc	Acetone biosynthesis	++	+	o	−	−	−	Lehmann et al. 2012a
Cac-*adc*180s::CT[b]	Adc	Acetone biosynthesis	+	+	+	−	o	−	Cooksley et al. 2012
pta::int(80)ΔRAM-*adc*::int(180)[b]	Pta and Adc	Acetate and acetone biosynthesis	−	++	++	−	−	−	Lehmann et al. 2012a
ctfA::int(352)[b]	Acetoacetyl-CoA:acyl-CoA transferase (CtfA)	Acetone biosynthesis	++	o	o	- -	−	−	Lehmann et al. 2012a
Cac-*ctfA*398s::CT[b]	CtfA	Acetone biosynthesis	+	++	++	- -	o	−	Cooksley et al. 2012
Cac-*ctfB*132s::CT[b]	CtfB	Acetone biosynthesis	+	++	++	- -	o	−	Cooksley et al. 2012
CKW[b]	CtfB	Acetone biosynthesis	+	+	uk	- -	−	−	Jang et al. 2012b
pta::int(80)ΔRAM-*ctfA*::int(352)[b]	Pta and CtfA	Acetate and acetone biosynthesis	−	++	++	- -	−	−	Lehmann et al. 2012a
CEKW[b]	Pta and CtfB	Acetate and acetone biosynthesis	−	+	uk	- -	−	−	Jang et al. 2012b
CBEKW[b]	Pta, Buk and CtfB	Acetate, butyrate and acetone biosynthesis	−	+	uk	- -	−	−	Jang et al. 2012b
hbd::int(69)[b]	3-Hydroxybutyryl-CoA dehydrogenase	Butyrate and butanol biosynthesis	−	- -	o	−	++	- -	Lehmann and Lütke-Eversloh 2011
PJC4AAD[b,d]	Aldehyde/alcohol dehydrogenase (AdhE) 1	Alcohol biosynthesis	+	+	uk	- -	+	−	Green and Bennett 1996
Cac-*adhE1*468s::CT[b]	AdhE1	Alcohol biosynthesis	+	++	o	−	−	-	Cooksley et al. 2012
Cac-*adhE2*696s::CT[b]	AdhE2	Alcohol biosynthesis	o	o	o	o	o	o	Cooksley et al. 2012
Cac-*bdhA*459s::CT[b]	Butanol dehydrogenase A	Alcohol biosynthesis	o	o	o	o	o	o	Cooksley et al. 2012
Cac-*bdhB*475a::CT[b]	Butanol dehydrogenase B	Alcohol biosynthesis	o	o	o	o	o	o	Cooksley et al. 2012
Cac-0059-261 s::CT[b]	Alcohol dehydrogenase CAP0059	Alcohol biosynthesis	−	−	+	+	+	+	Cooksley et al. 2012
HCEKW[c]	Pta, CtfB and AdhE1	Acetate, acetone and alcohol biosynthesis	−	++	uk	- -	−	- -	Jang et al. 2013c

Table 1 (continued)

Mutant strain of *C. acetobutylicum*	Inactivated protein	Pathway	Product pattern						Reference
			Acetate	Butyrate	Lactate	Acetone	Ethanol	Butanol	
HCBEKW[c]	Pta, Buk, CtfB and AdhE1	Acetate, butyrate, acetone and alcohol biosynthesis	+	++	++	- -	- -	–	Jang et al. 2014
HYCBEKW[c]	Pta, Buk, CtfB, AdhE1 and hydrogenase (HydA)	Acetate, butyrate, acetone, alcohol and hydrogen biosynthesis	+	++	+	- -	- -	–	Jang et al. 2014
SolRH[c,d]	Putative repressor of solventogenic genes (SolR)	Solventogenesis	+	o	uk	+	+	+	Harris et al. 2001
824 (solR$^-$)[b]	SolR	Solventogenesis	uk	uk	uk	+	+	+	Shao et al. 2007
rex::int(95)[b]	Redox-sensing transcriptional regulator	Solventogenesis	o	–	o	–	++	+	Wietzke and Bahl 2012

Similar mutants are listed according to their publication date. Phenotypic characteristics refer to the respective control strains in batch cultivations: +, increased; ++, highly increased (≥+100 %); o, unchanged; –, reduced; - -, none; uk, unknown

[a] pH-5.5-controlled batch fermentation

[b] Without pH control batch fermentation

[c] pH-5.0-controlled batch fermentation

[d] Knockout mutants obtained by integrational plasmids; all other mutants were generated by using the mobile group II intron technology

revealed increased butyrate concentrations, and in some cases significant lactate formation, reflecting the metabolic complexity and general difficulty to manipulate the fermentative pathways by systematic means (Lehmann et al. 2012a; Jang et al. 2012b).

The most notable achievement of the newly developed methods for systematic genetic engineering was probably the comprehensive study by Jang et al., in which multiple knockouts and combinations thereof were constructed and characterized (Jang et al. 2012b). The *C. acetobutylicum* strains BBEKW and CBEKW represent the first examples of distinct triple-knockout mutants with defects in the acetate, butyrate and acetone formation pathways (Table 1). In another publication, the mutant strain CEKW with inactivated Pta and CtfB was converted to the butyrate producer HCEKW by additional disruption of the *adhE1* gene to diminish solvent formation, yielding the third triple-knockout mutant of *C. acetobutylicum* (Jang et al. 2013c). Very recently, the same authors reported on the generation of novel quadruple and quintuple *C. acetobutylicum* mutants for improved butyrate production (Jang et al. 2014). It is noteworthy that this work included the first successful knockout of the hydrogenase gene *hydA*. Inactivation of *hydA* in the wild-type strain has been attempted by different laboratories (unpublished results, Cooksley et al. 2012), but obviously, this targeted mutation requires a particular genetic background as given by disrupted *pta*, *buk*, *ctfB* and *adhE1* genes in the HCBEKW strain (Table 1).

With respect to high-butanol yields, ethanol also constitutes an unwanted by-product, but elimination of ethanol formation by rational means seems to be a currently unsolvable problem, because ethanol and butanol are synthesized by the same enzymes. In order to assess the roles of the different alcohol dehydrogenases encoded in the genome of *C. acetobutylicum*, Cooksley et al. generated different alcohol dehydrogenase-deficient mutants using the ClosTron mutagenesis system (Cooksley et al. 2012). Among these mutants, only the AdhE1-deficient strain revealed a clear phenotype with drastically reduced solvent production and significant acid accumulation (Table 1). Interestingly, inactivation of the second functionally active aldehyde/alcohol dehydrogenase AdhE2 did not result in any product pattern alterations, although a putative role in redox-dependent solventogenesis was proposed earlier (Fontaine et al. 2002; Hönicke et al. 2012; Wietzke and Bahl 2012).

Understanding the regulatory mechanisms of the solventogenic shift is of great interest, and identification of the molecular triggering machinery still remains an obstacle. The master sporulation factor Spo0A plays a central role in the clostridial life cycle, but the phosphorylation pattern differs from the well-studied *Bacillus* homolog, and recent work on various sigma factors broadened our knowledge substantially (Paredes et al. 2005; Bi et al. 2011; Jones et al. 2011; Steiner

et al. 2011; Tracy et al. 2011; Al-Hinai et al. 2014). With respect to solvent formation, disruption of the putative regulator SolR using the TargeTron technology clearly increased the ABE titre, which was in accordance with the phenotype of the SolR-deficient mutant described earlier (Harris et al. 2001; Shao et al. 2007). More recently, the redox-sensing transcriptional regulator Rex was shown to be de facto involved in the solventogenic shift, and disruption of the *rex* gene resulted in an enhanced alcohol production accompanied by low-acetone levels (Wietzke and Bahl 2012) (Table 1).

Engineering IBE production

Among the solvents produced by *C. acetobutylicum*, acetone represents the least attractive by-product, because in contrast to ethanol and butanol, it cannot be used as a biofuel. Therefore, in vivo reduction of acetone to isopropanol converts the native ABE fermentation to isopropanol-butanol-ethanol (IBE) fermentation, an alcohol mixture suitable for direct utilization as fuel blend without previous separation of the solvents. This objective was pursued by four different research groups using different *C. acetobutylicum* strains or strain backgrounds, respectively. The key metabolic engineering step was the heterologous overexpression of the primary/secondary alcohol dehydrogenase gene *sadh* from *C. beijerinckii*. Using the *buk*-deficient strain PJC4BK as host, expression of *sadh* and homologous overexpression of *adc* and *ctfAB*, encoding the acetone biosynthetic pathway, resulted in 20 g/l IBE in batch fermentations (Lee et al. 2012). Later on, this group employed *C. acetobutylicum* BKM19, a strain preengineered for ABE production (see "Phenotype screening" section), as host strain and achieved 28 g/l IBE in lab-scale batch fermentations. In addition, the applicability of this recombinant strain for large-scale IBE production was demonstrated by reproducing the lab-scale fermentation performance at a 200-l pilot scale (Jang et al. 2013a).

Dusséaux et al. also chose a host strain with impaired butyrate biosynthesis, *C. acetobutylicum* 824Δ*buk*, for overexpression of *sadh* plus *adc* and *ctfAB* and evaluated different expression levels using the native *thl* and *ptb* promoters. Application of the *ptb* promoter, which is highly active during the exponential growth rather than in the stationary phase, resulted in a final biofuel titre of 21 g/l. Moreover, the authors concluded that the elevated intracellular acetate concentrations and the high K_M value of the CtfAB enzyme for acetate constitute the major bottlenecks for IBE production in *C. acetobutylicum* (Dusséaux et al. 2013).

Heterologous overexpression of *sadh* in the wild-type *C. acetobutylicum* ATCC 824 yielded 16 g/l IBE, and combination with the native *adc* and/or *ctfAB* genes further increased the total alcohol titre with *ctfAB* overexpression showing the more significant effect. Overexpression of all three acetone biosynthetic genes along with the heterologous *sadh* gene yielded 24 g/l IBE in addition to small amounts (1.2 g/l) of 2,3-butanediol (Collas et al. 2012). Interestingly, the same IBE titre was reported by Dai et al., who introduced the *sadh* gene into the preengineered mutant strain *C. acetobutylicum* Rh8 (see "Random mutagenesis" section) without concomitant *adc* and *ctfAB* overexpression (Dai et al. 2012). Chromosomal integration of *sadh* into the genome of *C. acetobutylicum* ATCC 824 led to an isopropanol titre of 1.7 g/l, demonstrating the general feasibility of the ACE technique to construct plasmid-independent overexpression strains (Heap et al. 2012).

Engineering substrate utilization

Utilization of cheap non-food feedstocks is the ultimate prerequisite for economically feasible biofuel production (Gu et al. 2011; Tracy et al. 2012; Xue et al. 2013). Since solventogenic clostridia can metabolize a wide variety of hexose and pentose sugars, they are predestined to convert agricultural waste products in the form of pretreated lignocellulosic hydrolysates to bulk chemicals or fuels (Jurgens et al. 2012). In order to promote simultaneous and efficient utilization of these carbohydrate mixtures, metabolic pathways and their regulatory circuits must be known for sustainable metabolic engineering strategies. Components of the phosphoenolpyruvate-dependent phosphotransferase systems (PTS) are suitable targets to enable cofermentation of glucose, xylose and arabinose, the three dominant sugars of lignocellulosic biomass. The glucose PTS of *C. acetobutylicum* has been identified, and inactivation of the enzyme-II-encoding *glcG* gene alleviated the glucose-dependent repression of xylose catabolism without affecting glucose utilization (Tangney and Mitchell 2007; Xiao et al. 2011). Additional overexpression of the *xylTBA* genes, coding for xylose-proton symporter, xylose isomerase and xylulose kinase, in the *glcG*-negative mutant increased the ABE production by 24 % from a glucose-xylose-arabinose mixture (Xiao et al. 2011). Employing the industrial strain *C. acetobutylicum* EA 2018 as host for the same genetic modifications, a greater increase of 44 % of the ABE titre was reported (Li et al. 2013). Further, potential pentose phosphate pathway genes were identified in the genome of *C. acetobutylicum*, and the respective functionalities were experimentally validated. Overexpression of the four genes encoding transketolase, transaldolase, ribose-5-phosphate isomerase and ribulose-5-phosphate epimerase significantly improved xylose utilization, yielding a 42 % higher solvent titre from xylose as compared to the plasmid control strain (Jin et al. 2014). However, xylose and arabinose are not exclusively metabolized via the pentose phosphate pathway; the existence of a functional phosphoketolase pathway in *C. acetobutylicum* was recently demonstrated by two independent studies (Liu et al. 2012a; Servinsky et al. 2012).

Cofermentation of glucose and xylose was also achieved by disruption of the *ccpA* gene, encoding the catabolite control protein A, a pleiotropic regulator of carbon catabolite repression. But since CcpA controls a large variety of genes, the CcpA-negative mutant accumulated elevated amounts of acids in pH-uncontrolled cultures, which in turn negatively affected cell growth and solvent formation (Ren et al. 2010). In a follow-up publication, the global role of CcpA was assessed in a detailed transcriptional profiling approach. Interestingly, CcpA does not only control genes involved in sugar uptake and metabolism; solventogenic and sporulation-associated genes are also regulated by CcpA (Ren et al. 2012).

Finally, possible glycolytic limitations in *C. acetobutylicum* were addressed by homologous overexpression of the *pfkA* and *pykA* genes, coding for 6-phosphofructokinase and pyruvate kinase, which increased the final ABE titre by 23 % in batch cultures (Ventura et al. 2013).

Explorative metabolic engineering

Random mutagenesis

In general, the systematic metabolic engineering strategies described above implement the rational design and analysis of particular genetic alterations derived from gene overexpression and/or inactivation. To accomplish this, two indispensable prerequisites must be fulfilled: (i) physiological and genetic knowledge for the ability to define targets and (ii) availability of molecular tools to introduce specific manipulations. In contrast, random mutagenesis can bypass these constraints and broadens the opportunities to obtain useful strains for ABE fermentation independently from sophisticated techniques. Thus, traditional strain breeding was—and still is—quite successful to isolate *C. acetobutylicum* mutants with enhanced phenotypes. Most commonly, chemical agents such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine or ethyl methane sulphonate were used rather than ultraviolet irradiation, which is less effective for clostridia as compared to Gram-negative bacteria, to generate respective mutant populations (Bowring and Morris 1985; Lemmel 1985). Today, less harmful methods are desirable to protect laboratory personnel from exposure to toxic and carcinogen substances and reduce the use of polluting agents. Hence, ionized gas beams for mutagenesis might substitute highly toxic chemicals, and nitrogen ion beam implantation (NIBI) as well as atmospheric and room temperature plasma (ARTP) was shown to generate suitable *C. acetobutylicum* mutants (Liu et al. 2012b; Li et al. 2014).

Genome shuffling, an evolutionary method based on recursive genomic recombination, allows rapid phenotype improvements. In comparison to conventional strain breeding, genome shuffling requires fewer sequential steps to obtain an optimal phenotype because of minimizing deleterious mutations and maximizing genetic exchange between parental strains (Zhang et al. 2002; Biot-Pelletier and Martin 2014). This two-step procedure of chemical mutagenesis plus selection to generate appropriate parents and subsequent recursive protoplast fusion was shown to generate improved ABE producing mutants of *C. acetobutylicum*, including strain Rh8 which was subjected to detailed comparative proteomics (Mao et al. 2010, 2011; Gao et al. 2012b).

A more complex synthetic system to generate potential mutant libraries of *C. acetobutylicum* was developed by Luan et al., and advantageously, the degree of mutagenesis can be adapted easily. For this, the chromosomal mismatch repair operon *mutSL* was inactivated and complemented by a plasmid harbouring *mutSL* alleles fused to the anhydrotetracycline-inducible Pcm-2tetO1 promoter as well as two copies of the TetR repressor gene to ensure stringency. Thus, the mutation rates during genome replication can be modulated up to 120-fold as compared to the wild-type by controlling the inducer concentration (Luan et al. 2013).

Phenotype screening

Basically, various options to create mutant libraries of *C. acetobutylicum* exist, and transfer of novel methods successfully developed for other bacteria such as promoter or transcription factor engineering should be transferable to clostridial species (Kim et al. 2013). Such libraries can be based on single or multiple genomic mutations, mutated global regulators or other specific proteins, in addition to overexpression libraries comprising homologous or heterologous genomic DNA fragments (Borden and Papoutsakis 2007; Borden et al. 2010). However, not the type of library but the availability of new screening methods is currently limiting the application of explorative metabolic engineering strategies. In order to identify desired phenotypes from large populations, the screening method must be conducted in a high-throughput manner, prioritizing technical feasibility, i.e. general handling, expenditure of work, time and costs. Since microbial biofuels are not directly detectable without chromatographic measurements, they must somehow be visualized by colorimetric/fluorescent methods or growth-dependent assays (Dietrich et al. 2010). With the few exceptions listed below, all *C. acetobutylicum* strains originating from explorative approaches reported so far were isolated due to survival of high butanol concentrations. The phenotype of improved tolerance was generally associated with increased ABE production as compared to the respective parental strains (e.g. Mao et al. 2010; Gao et al. 2012a; Liu et al. 2012b). A possible drawback of randomly mutagenized strains exhibiting improved tolerance and/or solvent production is the unknown genotype. It is self-evident that an ideal butanol-tolerant phenotype is usually determined by multiple

genetic alterations, and backtracking these mutations is required to identify novel and interesting target genes for both understanding and engineering cellular robustness towards solvents (Jia et al. 2010; Nicolaou et al. 2010).

Strategies to investigate and modify metabolic pathways during the pregenome era of *C. acetobutylicum* often included the use of compounds mimicking natural substrates or intermediates. For example, antimetabolites are useful non-metabolizable agents to select deregulated mutants for the overproduction of primary metabolites like amino acids (Lütke-Eversloh and Stephanopoulos 2005; Sanchez and Demain 2008). Suicide substrates represent another group of practical compounds, and since only the cleavage products are toxic, their use allows a positive selection of clones which lost the ability of degradation and thus isolation of mutants with particular enzymatic defects. Employing halogenated carboxylic acid analogues, various mutants of *C. acetobutylicum* with altered metabolite profiles were isolated (e.g. Junelles et al. 1987; Clark et al. 1989; Medkor et al. 2010). Uptake and conversion of fluoroacetate results in the formation of the highly toxic intermediate fluoroacetyl-CoA and therefore clones able to grow in the presence of fluoroacetate possess mutations in the acetate metabolic pathway (Rothstein 1986). This principle was recently employed by S. Y. Lee and coworkers who generated *C. acetobutylicum* BKM19. Interestingly, this was the first example of combined systematic and explorative metabolic engineering: The preengineered Buk-negative strain PJC4BK (Table 1) was subjected to chemical mutagenesis, and the resulting library was screened for growth on fluoroacetate-containing agar plates. The selected mutant BKM19 exhibited a significantly improved phenotype; i.e. the ABE titre of batch cultures was increased by 31 and 91 %, as compared to the parental PJC4BK strain and the wild-type, respectively (Jang et al. 2013b).

The peculiarity of the third strategy of phenotype selection is its direct association with alcohol synthesis instead of analysing derived parameters commonly attributed to enhanced butanol formation, for instance, high tolerance, defects in competing pathways or particular cell morphologies. In order to visualize butanol and ethanol in clostridial microtitre cultures, a colorimetric assay using nitroblue tetrazolium derivatization was developed to establish a high-throughput screening system for biofuels. Screening of two chemically mutagenized *C. acetobutylicum* libraries yielded several mutants with >20 % increased butanol production. On the one hand, this semi-quantitative method was coupled with a preselection step employing butanol tolerance as parameter to reduce the library size for the subsequent microtitre-based screening. On the other hand, a preengineered strain with disrupted *adc* gene and thus low-acetone production, was used as the parental strain for the library, yet another example of successful implementation of systematic and explorative engineering approaches (Scheel and Lütke-Eversloh 2013).

Finally, another strategy took advantage of the relation between metabolic states and different cell morphologies typically associated with clostridial sporulation. High-throughput flow cytometric analyses comprising different staining methods were used to discriminate and quantify various cell types of *C. acetobutylicum* (Tracy et al. 2008). Establishment and optimization of single-cell analysing methodologies offer valuable options to investigate basic research objectives such as the correlation of morphological and physiological phenotypes (Tracy et al. 2010). Moreover, flow cytometry was proposed to be a useful tool for process mapping of clostridial ABE fermentation, including differentiation of acidogenic and solventogenic subpopulations and determination of cell viability (Linhová et al. 2012; Patáková et al. 2013).

In summary, four phenotype screening principles are currently available for *C. acetobutylicum* to select improved biofuel producing mutants: (i) butanol tolerance, (ii) suicide substrates, (iii) colorimetric alcohol detection, and (iv) flow cytometry. Future research will certainly expand this portfolio of high-throughput screening strategies, and researchers can learn from promising methods developed for other microorganisms. For example, a butanol-specific biosensor was recently developed to monitor butanol formation in recombinant *E. coli* cultures. The transcription-factor-based system was composed of the σ^{54}-dependent activator BmoR and the alcohol-controlled promotor P_{BMO} from the alkane-degrading β-proteobacterium *Thauera butanivorans* (formerly *Pseudomonas butanivorans*), fused to the tetracycline resistance gene *tetA*. This biosensor exhibited a linear correlation of *E. coli* growth and 1-butanol concentrations in the range of 0.01–40 mM, whereas ethanol was not recognized (Dietrich et al. 2013).

Enzyme engineering

Although solventogenic clostridia represent industrially relevant microbes with unique metabolic capacities, only little work has been conducted on specific enzyme engineering, except for cellulose-degrading proteins (Kellermann and Rentmeister 2014; Thomas et al. 2014; Yang et al. 2014). Most of the central fermentative enzymes have been purified and biochemically characterized (Gheshlaghi et al. 2009), but only two studies on engineering key enzymes of ABE fermentation were published recently. In a rational approach, Jang et al. optimized the AdhE1 activity in the background of strain *C. acetobutylicum* BEKW (Table 1) for further enhancement of the carbon flux from acetyl-CoA to butanol via butyryl-CoA. According to sequence homologies to the *Zymomonas mobilis* alcohol dehydrogenase 2, the cofactor specificity of the clostridial AdhE1 was attenuated by a simple D-485-G amino acid substitution, allowing both NADPH and NADH to be used as electron donors (Rellos et al. 1997; Jang et al. 2012b).

Based on the idea of alleviating feedback inhibition of the branchpoint enzyme of the C_4 biosynthetic pathway, the thiolase of *C. acetobutylicum* was specifically engineered to reduce the sensitivity towards its physiological inhibitor free CoA-SH. First, a microtitre high-throughput assay was developed to analyse thiolase activities and CoA-SH inhibition in recombinant *E. coli* cell extracts. Subsequently, a mutant library of the clostridial *thlA* gene was generated, whereby only the region of CoA-binding was mutagenized with an average mutation rate of 2.5 amino acid substitutions. Screening of this library resulted in the identification of thiolase derivatives with a more than tenfold decreased inhibition by 50 μM CoA-SH, which is clearly below the physiological concentration (Wiesenborn et al. 1988). Finally, overexpression of the engineered thiolase gene in *C. acetobutylicum* resulted in an 18 % higher butanol titre and a significant delay in ethanol and acetone production (Mann and Lütke-Eversloh 2013).

The thiolase engineering approach belongs to the explorative category of metabolic engineering; however, recombinant *E. coli* cells were used to facilitate the screening procedure. With respect to future attempts, genetic engineering might also be performed directly in *C. acetobutylicum* as the native host. Introduction of the *Clostridium perfringens* phagenic *recT* gene was recently shown to mediate recombination of synthetic oligonucleotides, i.e. single-strand DNA, in *C. acetobutylicum*. Further optimization of this method might enable genomic recombineering in the near future and thus expand the opportunities for single-protein engineering or even whole-cell engineering (Dong et al. 2014).

Concluding remarks

The metabolic engineering toolbox for *C. acetobutylicum* and related solventogenic clostridia has thrived enormously in the past few years, and application of modern genetic techniques has eventually led to competitive results as compared to classical strain breeding. There are several pros and cons relating to whether butanol production is more attractive in native clostridia or in alternate host organisms (Gronenberg et al. 2013; Branduardi et al. 2014). Since redox-mediated limitations such as the non-functionality of the ferredoxin-dependent butyryl-CoA dehydrogenase complex (Bcd/EtfAB) in *E. coli* have been resolved, heterologous butanol production in well-accessible host organisms is definitely reasonable, and even in vitro synthesis of butanol from glucose is possible (Li et al. 2008; Bond-Watts et al. 2011; Shen et al. 2011; Lim et al. 2013; Krutsakorn et al. 2013). On the other hand, the recent progress in developing applicable molecular technologies for solventogenic clostridia again compensates for the potential advantages of non-native hosts. Together with the bioinformatic networks providing steadily increasing amounts of 'omics' data as well as complex *in silico* models, the availability of suitable experimental protocols for genetic modifications is pivotal for the application of systems biology to effectively engineer *C. acetobutylicum* (Jang et al. 2012d; Woolston et al. 2013).

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