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MINI-REVIEW

New technologies provide more metabolic engineering strategies for bioethanol production in *Zymomonas mobilis*

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Abstract

Bioethanol has been considered as a potentially renewable energy source, and metabolic engineering plays an important role in the production of biofuels. As an efficient ethanol-producing bacterium, *Zymomonas mobilis* has garnered special attention due to its high sugar uptake, ethanol yield, and tolerance. Different metabolic engineering strategies have been used to establish new metabolic pathways for *Z. mobilis* to broaden its substrate range, remove competing pathways, and enhance its tolerance to ethanol and lignocellulosic hydrolysate inhibitors. Recent advances in omics technology, computational modeling and simulation, system biology, and synthetic biology contribute to the efficient re-design and manipulation of microbes via metabolic engineering at the whole-cell level. In this review, we summarize the progress of some new technologies used for metabolic engineering to improve bioethanol production and tolerance in *Z. mobilis*. Some successful examples of metabolic engineering used to develop strains for ethanol production are described in detail. Lastly, some important strategies for future metabolic engineering efforts are also highlighted.

Keywords Bioethanol · Metabolic engineering · *Zymomonas mobilis* · Lignocellulosic hydrolysates · Inhibitor

Introduction

The use of biofuels is regarded as a promising solution to the challenges of energy security, urban air quality, and CO_2 emissions and so on. Currently, fuel ethanol (C_2H_6O) is used in China and other countries in the form of a 10% additive to other fuels. In general, bioethanol can be produced from various microbes, such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Z. mobilis*, *Clostridium thermocellum*, *Klebsiella oxytoca*, and *Clostridium acetobutylicum* by fermenting renewable resources such as energy-rich crops or lignocellulosic biomass (Fischer et al. 2008). Of these microbes, *E. coli* has been the most widely used for metabolic engineering due to its rapid growth and many available genetic engineering tools. *S. cerevisiae* has been the most widely employed eukaryotic microbe for ethanol fermentation. As a model ethanologenic strain, *Z. mobilis* has attracted considerable attention due to its unique characteristics, including its high rate of sugar uptake, high ethanol yield, high tolerance to glucose (up to 400 g/L), high resistance to ethanol (up to 16% *v*/*v*), and its generally regarded as safe (GRAS) status. Furthermore, *Z. mobilis* uses the Entner-Doudoroff (ED) pathway to produce ethanol, resulting in low cell mass and a high transformation efficiency (up to 98% of the theoretical ethanol yield) (Rogers et al. 2007; Swings and De Ley 1977). In addition, *Z. mobilis* has been developed as an excellent microbial chassis strain for the production of biofuels and biochemicals, including sorbitol, isobutanol, gluconic acid, and levan (He et al. 2014).

Although native *Z. mobilis* strains have high efficient enzyme system, it is quite difficult to increase the ED flux through overexpression of glycolytic enzymes for bioethanol production, even to be unsuccessful (Rutkis et al. 2013). Traditionally, random mutagenesis was adopted to obtain strains producing higher amounts of ethanol. However, the efficiency of this approach is low due to the generation of undesired alterations in the genome and the unpredictable consequences. Thus, the use of rational engineering strategies is necessary, especially for targeting specific genes and pathways. Metabolic engineering methods are being increasingly used to improve microbial bioethanol production and other types of biofuels. Furthermore, recent advances in system

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biology, synthetic biology, evolutionary engineering, and genome editing also contribute to construction and modification of ethanol-producing pathways in *Z. mobilis*.

General metabolic engineering strategies for bioethanol production

Titer, yield, and productivity are the primary fermentation performance metrics used for almost all microbially produced chemicals (Lee et al. 2009). Lignocellulosic biomass must be hydrolysed to disaccharides and monosaccharides, which are subsequently converted into ethanol by different microbes. However, this process simultaneously generates diverse inhibitors, such as furfural, 5-hydroxymethyl-2-furaldehyde (HMF), acetate, and vanillin. Suitable engineered strains must tolerate various stresses, such as ethanol and inhibitors, to achieve high yields and titers. Metabolic engineering strategies used to produce various biosynthetic chemicals are also valid for increasing ethanol production. Some basic genetic and computational tools and optimization strategies for metabolic pathways and networks are essentially the same as those used to increase ethanol production in *Z. mobilis*.

The difficulties associated with metabolic engineering in *Z. mobilis* involve the following aspects: the narrow substrate range, competition of the bypass pathway, the cytotoxic effect of ethanol and the released inhibitors from lignocellulosic hydrolysates, the inefficient simultaneous saccharification, and fermentation process caused by the low heterologous expression level of cellulase, and the poor pentose (e.g., xylose and arabinose) utilization capability resulting from the glucose effect or carbon catabolite repression (CCR), among others. To solve these problems, various microbial metabolic engineering strategies have been used, and significant progress has been made in recent years. Currently, these metabolic engineering strategies primarily follow the principle of "broaden sources of income and reduce expenditure." Several excellent reviews have been published and focus on description of historical milestones of *Z. mobilis* development, especially with respect to the expansion of substrate utilization, the production of ethanol and other biochemicals, and improved tolerance (He et al. 2014; Panesar et al. 2006; Rogers et al. 2007; Wang et al. 2018; Yang et al. 2016; Yang et al. 2018). In this review, we discuss significant progress that has allowed for the successful metabolic engineering of *Z. mobilis* with respect to ethanol production.

The integration of various omics data from different physiological statuses

Traditional metabolic engineering is more effectively employed when used together with omics techniques and computational modeling and simulation. With the widespread application of genomics, transcriptomics, proteomics, metabolomics, and fluxomics, it is feasible to identify key elements and regulators under different conditions from whole genome-wide analyses that can serve as new engineering targets to re-design and construct new potential pathways in host strains. The genomes of ZM4 and other *Z. mobilis* cultivars have been reported, facilitating the application of microarray approaches (Seo et al. 2005; Yang et al. 2009a). For example, a comparative transcriptome analysis between two *Z. mobilis* strains (ZM4 and ZM1) identified four highly expressed ORFs that are potentially related to the higher rates of glucose uptake and ethanol production in the ZM4 strain and two genes encoding capsular carbohydrate synthesis enzymes that were closely associated with osmotic pressure resistance at high glucose and ethanol concentrations (Seo et al. 2005). These genes could be potential target for reverse engineering to improve the tolerance of strains to high concentrations of glucose and ethanol.

Transcriptome data are useful to analyze the physiological differences between strains and understand the underlying genetic mechanisms. Transcripts for ED pathway genes (*glk*, *zwf*, *pgl*, *pgk*, and *eno*) and the pyruvate decarboxylase (PDC)–encoding gene *pdc* were observed to be more abundant under anaerobic conditions than aerobic conditions in the strain ZM4 (Yang et al. 2009b). In addition, the molecular mechanisms of *Z. mobilis* adaptation to high glucose concentrations was investigated using a DNA microarray, and ZM4 was observed to respond to high concentrations of glucose by modulating the transcriptional levels of genes associated with membrane channels and transporters, stress response mechanisms, and ED pathways (Zhang et al. 2015).

Both ethanol and the inhibitor acetate derived from lignocellulose pretreatment have a negative effect on the growth rate, glucose consumption, and energy maintenance of *Z. mobilis*. Transcriptomic studies demonstrated that the response of ZM4 to ethanol is a dynamic and complex process involving many different functional genes, as demonstrated by a transcriptomic study of *Z. mobilis* under 5% ethanol stress (He et al. 2012a; Yang et al. 2013). Additionally, transcriptome data revealed that several reductases, encoded by *ZMO1116*, *ZMO1696*, and *ZMO1885*, play key roles in the response of ZM4 to phenolic aldehyde inhibitors, as these enzymes can convert phenolic aldehydes into phenolic alcohols (Yi et al. 2015). In another study, a TonB-dependent receptor gene (*ZMO0128*) knockout mutant was shown to exhibit acetate tolerance in the presence of different substrates (Yang et al. 2014b). In addition, the combined results derived from transcriptomics and quantitative proteomics indicated that minimal medium had the most significant effect on gene expression compared to rich medium, followed by growth phase, inhibitor, and strain background (Yang et al. 2014a). Three regulatory sRNAs were shown to be differentially expressed under aerobic/anaerobic and 5% ethanol stress

conditions based on the results of a transcriptome analysis and computational predictions (Cho et al. 2014). The proteomic results revealed that there was no significant difference in the stress response to toxic inhibitors of biofilm-associated and planktonic *Z. mobilis* ZM4 cells (Todhanakasem et al. 2018). In addition, metabolomic profiles of ZM4 cultured aerobically and anaerobically showed that oxygen can lead to an increase of metabolic by-products, such as acetate, lactate, and acetoin (Yang et al. 2009b). Bochner et al. reported that *Z. mobilis* ZM4 was relatively resistant to an acidic pH (approximately 4.0) and various inhibitory chemicals using Phenotype MicroArray™ profiling (Bochner et al. 2010). The toxic effects of model inhibitors found in hydrolysate on the growth of *Z. mobilis* 8b was investigated and the result showed that these typical inhibitors did not interact in a synergistic manner, which was different from *E. coli* and *S. cerevisiae* (Franden et al. 2013).

The results of these studies demonstrated that multiple genes involved in carbohydrate metabolism, cell membrane synthesis, recombination and repair, HSPs, and the universal stress responses, among others, are engaged in responding to different substrates and inhibitors in *Z. mobilis*. These high-throughput omics methods contribute to systematic analyses that further our understanding of cellular responses to different conditions. In addition, different inhibitory profiles aid in identifying the contributions of different inhibitory components of lignocellulosic hydrolysates and provide guidance for potential process development and strain improvement and tolerance engineering strategies. These profiles can subsequently be used to identify new targets for the metabolic engineering modifications discussed below.

Computational modeling and simulation for predicting metabolic engineering targets

When target genes are selected during traditional metabolic engineering, the interactions and dynamic regulations between various metabolic pathways are often neglected. However, there are complex underlying mechanisms that regulate the flux distribution in cells. Thus, the consequences of genetic manipulation are difficult to precisely predict and are frequently not obvious. Genome-scale modeling and in silico analysis are helpful for identifying appropriate gene targets to be engineered based on an evaluation of whole cellular system. This approach can predict the consequences of gene manipulation and environmental perturbations on cellular metabolism. In combination with other high-throughput techniques, this method has been successfully used to design strategies for engineering microorganisms to produce various value-added chemicals and fuels, including lycopene, L-valine, and ethanol (Alper et al. 2005b; Bro et al. 2006; Park et al. 2007). Various mathematical models were established to predict the performance of batch fermentation of mixtures of glucose and xylose, explain the physiological and metabolic characteristics, and suggest novel pathways to produce high-value-added products (Leksawasdi et al. 2001; Lee et al. 2010; Pentjuss et al. 2013). The generation of ED pentose phosphate pathway (PPP) network models for xylose-fermenting *Z. mobilis* strains allowed some possible ways of maximizing xylose conversion to be suggested, including avoiding enhancement of metabolic burden and the unproductive accumulation of intracellular metabolites (Altintas et al. 2006; Tsantili et al. 2007). These results can serve as good examples of the application of genome-scale metabolic models of *Z. mobilis*.

In addition, constraints-based flux analysis is another powerful tool for predicting metabolic engineering targets, as this approach can estimate the effects of gene knockouts on whole-cell metabolic flux distribution by setting the corresponding flux value to zero. The results of simulations showed that pyruvate decarboxylase and D-lactate dehydrogenase are the best two targets for a double gene knockout to promote succinic acid overproduction, which was in agreement with a previous study (Seo et al. 2007). Additionally, Widiastuti et al. reported that less carbon flux is diverted to the PPP and the tricarboxylic acid (TCA) cycle due to a deficiency of several genes. Meanwhile, two genes, *pdc* and *adh*, were recognized as being the most significant genes for ethanol production (Widiastuti et al. 2011). Thus, these models and simulations can enable the identification of reactions that are essential for cellular growth or ethanol production and the elucidation of the connectivity between the various pathways in a network. These results provide significant insight towards the design of future experiments and the generation of data. Combined with the simulations, these approaches can enhance our understanding of the regulation of ethanol production in *Z. mobilis*. Moreover, the knowledge gained from these analysis could also be applied to further improve the carbon source utilization, redox balance, and production of other desirable metabolites in *Z. mobilis*.

Broadening the substrate range

Constructing new pathways for substrate utilization

Wild-type *Z. mobilis* can only utilize glucose, fructose, and sucrose as carbon sources to produce ethanol. Nevertheless, it cannot utilize pentoses such as xylose, which is the second most abundant sugar in pretreated biomass hydrolysates. To expand the substrate range of *Z. mobilis*, various hydrolase-encoding genes from other species have been transferred into *Z. mobilis* strains, including an amyloglucosidase-encoding gene from *Aspergillus niger* (Skotnicki et al. 1983), an α-amylase-encoding gene from *Bacillus licheniformis* (Wang et al. 2012;

Brestic-Goachet et al. 1990); endoglucanase-encoding genes from *Bacillus subtilis* (Yoon et al. 1988), *Erwinia chrysanthemi* (Brestic-Goachet et al. 1989), and *Pseudomonas fluorescens*; and β-glucosidase-encoding genes from *Xanthomonas albilineans* (Su et al. 1989) (Fig. 1). However, no or low enzymatic activity was detected in the recombinant strains. These studies have laid the foundation for the construction of recombinant *Z. mobilis* that can ferment xylose directly. The carboxymethyl cellulase–encoding genes isolated from *Acetobacter xylinum* were heterologously expressed in *Z. mobilis*, leading to a tenfold greater level of gene expression than was observed in *E. coli* (Okamoto et al. 1994). When the D-xylose catabolic genes from *Xanthomonas campestris* were subcloned and introduced into *Z. mobilis*, the levels of xylose permease and xylose isomerase expression were 12- and twofold lower than was observed in the *X. campestris* donor strain, respectively. In 1995, Zhang et al. successfully constructed the recombinant strain *Z. mobilis* CP4 (pZB5), which can directly convert xylose to produce ethanol. This strain is capable of the simultaneous expression of xylose isomerase (XI), xylulokinase (XK), transaldolase (TAL), and transketolase (TKT) from *E. coli* (encoded by the genes *xylA*, *xylB*, *tktA*, and *talB*, respectively), achieving an ethanol yield of 86% of its theoretical value (Zhang et al. 1995) (Fig. 1). The L-arabinose metabolic pathway was also introduced into *Z. mobilis* by expression of five heterologous genes from *E. coli*, including *araA*, *araB*, and *araD*, which encode L-arabinose isomerase, L-ribulokinase, and L-ribulose-phosphate-4-epimerase, respectively, as well as *talB* and *tktA* (Deanda et al. 1996) (Fig. 1). To enhance their genetic stability and decrease the addition of antibiotics, all seven genes used for xylose and arabinose metabolism were integrated into the *Z. mobilis* genome (Mohagheghi et al. 2002). Other than glucose, xylose, and arabinose, *Z. mobilis* has also been imparted to the ability of fermenting mannose or galactose (Weisser et al. 1996; Yanase et al. 1991). These recombinant strains have been extensively summarized and are not further described here (Rogers et al. 2007; Wang et al. 2018).

The consolidated bioprocessing (CBP) approach, which can integrate enzyme production, enzyme hydrolysis, and sugar fermentation into a single microorganism, is considered to be a promising method for the cost-effective production of biofuels using lignocellulosic biomass (Parisutham et al. 2014). The β-glucosidase genes from *Ruminococcus albus* and *Bacillus polymyxa* were successfully expressed in *Z. mobilis* (Luo and Bao 2015; Yanase et al. 2005). Vasan et al. reported that the expression of endoglucanase from *Enterobacter cloacae* could produce 5.5% and 4% *v*/*v* of ethanol using carboxymethyl cellulose (CMC) and NaOH-pretreated bagasse, respectively (Vasan et al. 2011). Wu et al. constructed a secretion expression system consisting of α-amylase from *Bacillus amyloliquefaciens* and the native signal peptide of PhoD, which allowed for the hydrolysis of starch for ethanol production in *Z. mobilis* (Wu et al. 2014). A similar strategy was also adopted by Wang et al. (2012). In addition, Linger et al. successfully expressed two cellulolytic enzymes (E1 and GH12) in *Z. mobilis* that were cloned from *Acidothermus cellulolyticus* (Linger et al. 2010). The genes encoding endoglucanase, exoglucanase, and β-glucosidase from *Trichoderma reesei* were introduced into *Z. mobilis*, and the recombinant strain could produce 7–9.5% ethanol using different substrates after 72 h (Venkatesh 2015) (Fig. 1). Various cellulase genes from isolated gut bacteria of phytophagous insects and wood feeding termites have been

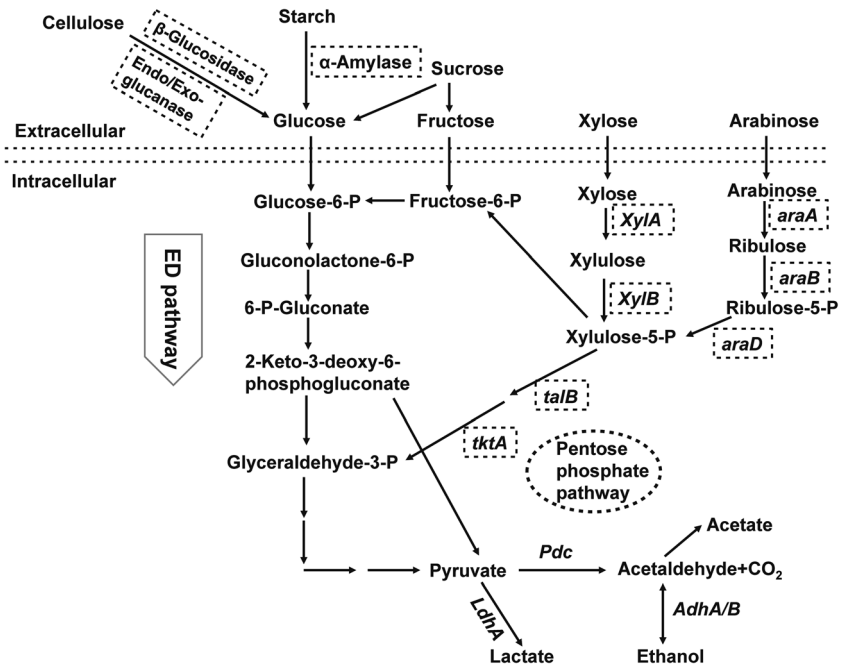

Fig. 1 Metabolic pathways for the conversion of various sugars into ethanol in *Z. mobilis*. Genes within the dotted box were introduced into *Z. mobilis* for establishing new metabolic pathways to broaden its substrate range. *XylA*, xylose isomerase; *xylB*, xylulokinase; *tktA*, transaldolase; *talB*, transketolase; *araA*, arabinose isomerase; *araB*, ribulokinase; and *araD*, ribulose-phosphate-4-epimerase

introduced into *Z. mobilis*, and all the resulting recombinant strains could directly ferment pretreated cellulosic substrates into ethanol (Haripriya and Vasan 2015; Misawa et al. 1988; Vasan et al. 2011). These studies contribute to the development of the CBP approach.

To satisfy the vitamin requirements for growth, a heterologous gene (*panD*) isolated from *E. coli*, which encodes an enzyme that catalyzes the production of β-alanine from aspartate to replace pantothenate (vitamin B_5), was introduced in *Z. mobilis* ZM4 to eliminate its pantothenate auxotrophy (Gliessman et al. 2017). Combined with utilizing N_2 as a nitrogen source (Kremer et al. 2015), this study demonstrated that it is possible to overcome the demand for nutrient-rich supplements for *Z. mobilis* growth on nitrogen-poor cellulosic feedstocks.

Improvement of xylose utilization efficiency

Compared with glucose, the ability to use xylose was observed to be significantly decreased by the presence of acetic acid in *Z. mobilis* (Kim et al. 2000). Xylose metabolism is often incomplete when its initial concentration is higher than 5%. An analysis of enzymatic activity profiles indicated that xylose isomerase may be a bottleneck in metabolically engineered *Z. mobilis* strains (Gao et al. 2002). To improve xylose utilization efficiency, XK-encoding gene, a strong terminator cluster T1-T2 of the *rrnB* rRNA operon, and xylose transporter gene *XylE* were introduced into the *Z. mobilis* strain, respectively (Jeon et al. 2005; Ma et al. 2012; Dunn and Rao 2014). The resulting recombinant strains showed no or limited increase in the rate of growth or xylose metabolism. However, the efficiency of xylose fermentation could be improved by adaptation and inverse metabolic engineering (Agrawal et al. 2011; Agrawal et al. 2012; Mohagheghi et al. 2014; Mohagheghi et al. 2015). The possible mechanism underlying the enhanced xylose metabolism is that xylose, the glucose analogue 2-deoxyglucose or pretreated corn stover hydrolysate, may exert a selective pressure similar to an antibiotic on parental strains. The results of these studies demonstrated that the classical strategy adaptation or continuous culture is still powerful tool for obtaining the desired phenotype. Recently, CRISPR/Cas9-facilitated multiplex pathway optimization (CFPO) was used to improve the xylose utilization pathway in *E. coli*, which can simultaneously regulate the expression of multiple genes associated of xylose pathway (Zhu et al. 2017). The use of this technique is an excellent example of the use of multiplex genome engineering to improve xylose utilization efficiency.

Attenuation and elimination of CCR

Owing to a mechanism known as CCR, most microbes, including *Z. mobilis*, preferentially utilize glucose over xylose. Engineered microorganisms capable of co-utilize glucose and xylose are of considerable interest to the biofuels industry, as they can simplify the fermentation processes and increase productivity. However, the elimination of CCR is a pressing challenge due to the multiple coordinating mechanisms involved, especially for those without any knowledge of their regulatory pathways. In previous studies, several strategies have been successfully used to circumvent CCR in *E. coli*, *Clostridium*, and *S. cerevisiae*, including overexpression of related genes participating in the xylose metabolic pathway (Yu et al. 2015), the mutagenesis of the pleiotropic regulator associated with CCR (e.g., CcpA) (Wu et al. 2015), the inactivation or mutagenesis of the sugar transporter system (Farwick et al. 2014), and the construction of an expression pathway for the direct use of cellobiose (Ha et al. 2015) and cellodextrin (Galazka et al. 2010). Notably, the cellobiose/cellodextrin utilization pathway was composed of a cellodextrin transporter and a β-glucosidase. In this system, the substrate cellobiose/cellodextrin is transported across the cell membrane by the cellodextrin transporter and is subsequently hydrolysed by β-glucosidase to release glucose. Because the intracellular glucose is quickly metabolized, CCR was eliminated.

Microbial consortia represents an alternative approach to circumvent CCR that mimics the degradation process of lignocellulosic plant biomass in nature. Currently, natural microbes from microbial consortia have been combined with engineered strains to form synthetic microbial consortia, which can compartmentalize different metabolic pathways into different hosts to accomplish complex tasks. For example, an *S. cerevisiae* and *E. coli* co-culture model was designed to produce ethanol in which wild-type *S. cerevisiae* only consumes glucose and the engineered *E. coli* strain ZSC113 only consumes xylose, avoiding the diauxic growth phenomenon commonly observed in pure cultures (Hanly et al. 2012). In addition, some rational algorithms can guide pathway optimization to overcome CCR. For example, the SIMUP algorithm was used to identify an optimum strategy to only allow *E. coli* to co-utilize glucose and xylose (Pratish et al. 2013). The major advantage of this method is that it does not require knowledge of regulatory mechanisms of CCR from the target microorganisms. These findings indicated that continuous culture or adaptation in combination with metabolic engineering can serve as a synergistic strategy for strain evolution engineering, contributing to improving the efficiency of xylose fermentation in *Z. mobilis* strains.

Blocking the competing or by-product synthesis pathways

The simplest strategy for inhibiting competing pathways is to decrease or shut down the undesirable metabolic pathways, increase the metabolic fluxes towards the desired product, and

balance the metabolic fluxes for optimal product formation. Blocking a pathway is often accomplished by deleting one or all genes at specific metabolic branch points. For the ZM4 strain, competing pathways include those that consume ethanol as precursors to generate other metabolites and utilize intermediates to yield by-products, such as acetate, acetoin, acetaldehyde, succinate, sorbitol, lactate, and xylitol, which compete with the synthesis of ethanol. For example, the lactate dehydrogenase gene *ZMO1237* (*ldhA*) was reported to be more abundant under aerobic and ethanol stress conditions as well as in the presence of high glucose concentrations (Yang et al. 2013; Yang et al. 2009b; Zhang et al. 2015). The upregulation of *ldhA* and a gene encoding D-lactate dehydrogenase (*ZMO0256*) may lead to the accumulation of lactate in *Z. mobilis*. Therefore, the deletion of the lactate dehydrogenase gene has a positive impact on the production of ethanol (Zhang et al. 2007). A similar promoting effect was also achieved via the inactivation of the *gfo* gene encoding glucose-fructose oxidoreductase (GFOR), which is responsible for the formation of the primary by-product sorbitol at high sucrose concentrations (Wang et al. 2013).

In many cases, competing pathways utilize essential enzymes for other metabolic pathways, and the deletion of the genes encoding these enzymes can cause severe phenotypes (e.g., auxotrophy or reduced energy generation) or be unsuccessful. For instance, several attempts have been made to delete the *pdc* gene, and the inability to obtain a mutant suggests that this gene is required for carbon metabolism (Widiastuti et al. 2011). Although the unique *pdc* and *adh* genes are required for efficient for ethanol production, the disadvantage of *pdc*-containing strains diverting the carbon from ethanol production to other desired products should be addressed. In contrast, engineering strategies that transiently control gene expression and block unwanted enzyme activity without gene deletion are advantageous. Recently, a method named TrimAway was developed to directly recognize and acutely degrade endogenous proteins without prior modification of the genome or mRNA, avoiding indirect protein depletion mediated by CRISPRi technology and RNA-targeting methods such as RNAi (Clift et al. 2017). Although this method has been used in mammal cells, whether it is suitable for bacteria requires further exploration.

Increasing cell tolerance to ethanol and inhibitors

The lignocellulosic inhibitors include weak acids (e.g., acetic acid), aldehydes (e.g., HMF, syringaldehyde, and furfural), and lignin degradation products (e.g., vanillin) (Franden et al. 2013). These inhibitors and ethanol are harmful to the integrity, fluidity, permeability, and lipid composition of the cell membrane, and the essential physiological processes, including nutrient transport, energy transduction, and mitochondrial stability (He et al. 2012b). The tolerance to single or multiple inhibitors present in hydrolysates are complex phenotypes and are controlled by undefined regulatory mechanisms. Furthermore, improving the tolerance of cells to inhibitors can involve many genes or metabolic nodes and may elicit a general stress response, which is also similar to ethanol tolerance (Nicolaou et al. 2010). The traditional domestication strategy can be used to obtain tolerant strains, but this process is time-consuming and labor-intensive. Moreover, the genetic traits of the resulting strains are not very stable. Therefore, to develop a strain with increased tolerance is often difficult due to the lack of detailed understandings of the tolerance mechanisms. In this regard, rational engineering and evolutionary engineering have been used to identify genetic targets associated with tolerance to develop more tolerant and robust strains.

Genes and regulatory elements involved in responding to ethanol and inhibitors

Hfq is a member of a conserved bacterial Sm-like family of RNA-binding proteins that are involved in coordinating regulatory responses to multiple stresses. Yang et al. demonstrated that the global regulator Hfq of *Z. mobilis* contributes to tolerance against multiple lignocellulosic pretreatment inhibitors, including acetate, vanillin, furfural, and HMF (Yang et al. 2010b) (Fig. 2). In addition, disruption of the *Z. mobilis gfo* gene resulted in a reduction in cell growth and ethanol production under osmolality, heat, and ethanol stresses (Sootsuwan et al. 2013). Agrawal et al. reported that xylose reductase from *Z. mobilis* exhibits nearly a 150-fold higher affinity for benzaldehyde than xylose. Therefore, this enzyme is able to mitigate furfural toxicity and that of other inhibitors from biomass hydrolysates (Agrawal and Chen 2011). A sodium acetate tolerance phenotype in a *Z. mobilis* AcR (acetic acid resistance) mutant was attributed to the overexpression of the sodium-proton antiporter gene *nhaA* (Yang et al. 2010a).

Additionally, 5′ untranslated regions (5′ UTRs) can function as regulatory elements to control the expression of mRNAs in response to various metabolites or environmental conditions. The 5′ UTR of the gene *ZMO0347*, which encodes the RNA-binding protein Hfq, was observed to downregulate the expression of downstream genes under ethanol stress in *Z. mobilis* (Cho et al. 2017). Another study showed that three sRNAs (Zms2, Zms6, and Zms18) were differentially expressed under 5% ethanol stress conditions, suggesting that these regulatory elements could be associated with regulatory mechanisms of ethanol production, tolerance, or stress responses in *Z. mobilis* (Cho et al. 2014) (Fig. 2). Interestingly, the result from genome resequencing of a mutant strain indicated that the enhanced xylose utilization efficiency may be resulted from a single nucleotide polymorphism (SNP) in the LysR-type transcriptional regulator-encoding

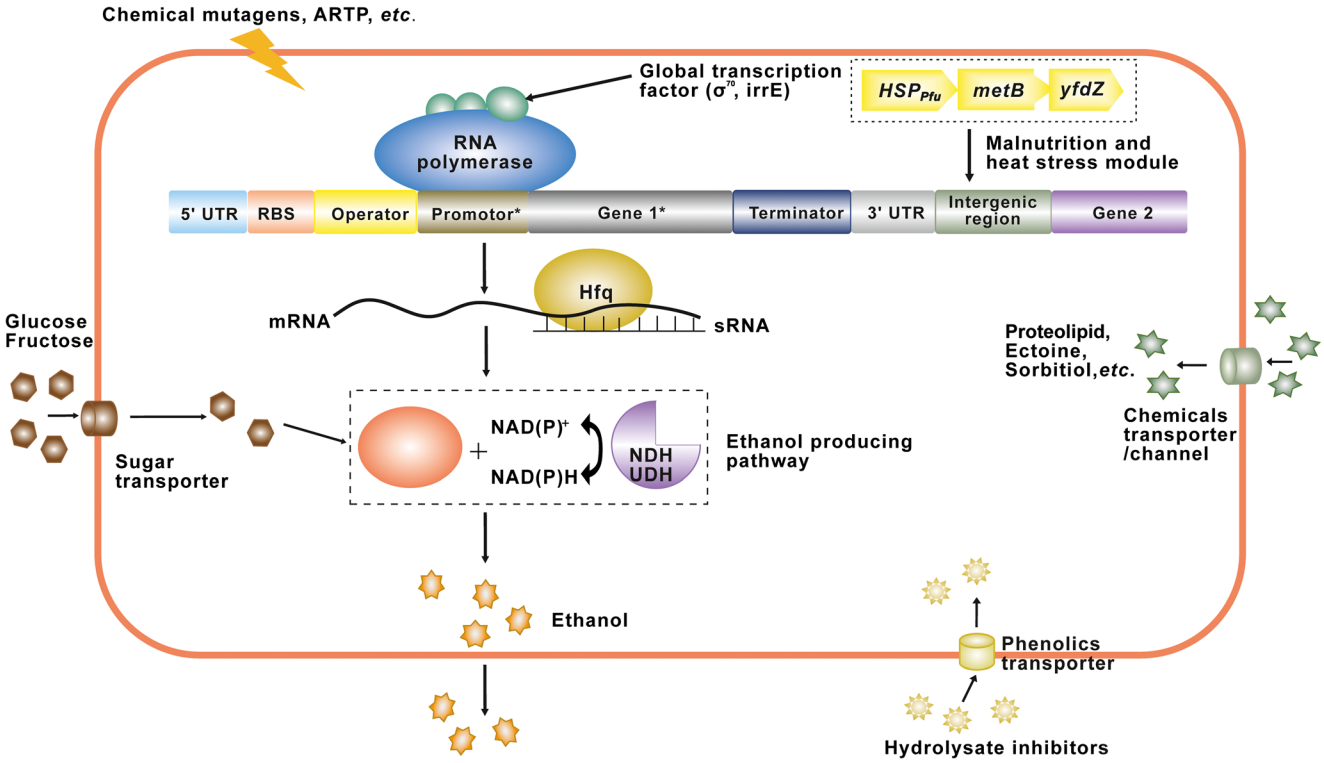

Fig. 2 Metabolic engineering strategies for improving tolerance to ethanol and inhibitors from pretreated lignocellulosic biomass. ARTP, the atmosphere and room temperature plasma; UTRs, untranslated regions; RBS, ribosome binding site; sRNAs, small RNAs; NDH, NAD(P)H dehydrogenase; UDH, transhydrogenase. Asterisk indicates a single nucleotide polymorphism (SNPs)

gene *ZMO0774* and several SNPs within the promoter region (Mohagheghi et al. 2014; Mohagheghi et al. 2015) (Fig. 2). Additionally, The result of this study revealed a strong role for altered transcriptional activities derived from changes in promoter activity, rather than protein mutations, lead to the enhancement of xylose utilization and ethanol productivity, suggesting that promoters can also serve as a potential targets for metabolic engineering. These studies can increase our understanding of the relationship between tolerance mechanisms and key genes. These newly discovered genes and regulatory elements can also serve as promising engineering targets for improving strain tolerance.

Modifications of multiple targets by mutagenesis and adaptive laboratory evolution

Chemical mutagenesis mediated by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) mutagen was initially used to mutate wild-type *Z. mobilis* to identify strains with different tolerances, resulting in the identification of a flocculating *Z. mobilis* strain ZM401, an acetate-tolerant strain (AcR), and a salt-tolerant strain (ZM482) that were isolated in succession (Joachimstahl et al. 1998; Lee et al. 1982; Rogers et al. 2007) (Fig. 2). Furthermore, the flocculating *Z. mobilis* mutant showed better tolerance to acetic acid and vanillin than the wild-type strain ZM4 (Zhao et al. 2014).

Adaptive laboratory evolution (ALE) is another powerful tool used to develop strains that are tolerant to lignocellulose inhibitors. For example, Shui et al. reported that a furfural- and acetic acid-tolerant strain was obtained by ALE. The best mutant ZMF3-3 achieved a 94.84% theoretical ethanol yield under a 3 g/L furfural stress condition, far more than the 9.89% yield from ZM4 (Shui et al. 2015). Given that furfural forms DNA-damaging free radicals in hydrolysates, the underlying mechanism may be that the hydrolysate itself is likely to act as a mutagen capable of creating genetic diversity for the selected strain. Further, *Z. mobilis* mutants with multiple resistance can be generated by ALE. For instance, a thermo-adapted strain (ZM AD41) showed not only higher thermotolerance but also more resistant to stress induced by acetic acid and hydrogen peroxide (H_2O_2) (Samappito et al. 2018). In addition, the ALE method can also be combined with NTG mutagenesis or transposon mutagenesis to effectively acquire the desired phenotypes (Liu et al. 2017). Recently, some novel mutagenesis methods, an adaptive evolution method based on the theory of stress-induced mutagenesis (SIM) and the atmosphere and room temperature plasma (ARTP) mutation method, have been shown to be powerful tools for phenotype improvement in many different strains (Cao et al. 2017b; Zhu

et al. 2014). These methods can also be applied in *Z. mobilis* to improve inhibitor tolerance.

Tolerance engineering

Although it is difficult to enhance tolerance by introducing only one or two genes, many efforts have been devoted to obtain the desired traits. Some key transcription factors, global regulatory proteins, and proteins involved in the general stress response have been used as engineering targets. For instance, *irrE*, a gene encoding a global regulator from *Deinococcus radiodurans*, can improve the tolerance of *Z. mobilis* to different inhibitors, such as ethanol, acid, osmotic stress, and thermal shock (Zhang et al. 2010) (Fig. 2). In addition, global transcription machinery engineering (gTME) can function as a useful tool to improve the ethanol and furfural tolerance of *Z. mobilis* by the random mutagenesis of the global transcription factor RpoD (σ^{70}) (Tan et al. 2015; Tan et al. 2016) (Fig. 2). These results suggest that global regulatory proteins such as RpoD can also serve as candidate targets of gTME or other protein-engineering techniques, and a similar strategy has also used for tolerance engineering for a broad range of stresses, such as low pH, osmotic and oxidative stress, and inhibitors (butanol, acetate, ethanol, and others).

Compatible solute sorbitol has been reported to play an important role in cell growth and ethanol fermentation in *Z. mobilis* under heat, ethanol, and osmotic stresses (Sootsuwan et al. 2013). Another compatible solute, ectoine, can also have an osmoprotection role under high glucose concentrations (Zhang et al. 2008). Furthermore, some components of the cell membrane such as proteolipids also show a similar effect on the ethanol fermentation of *Z. mobilis* (Weir and Chase 1995). Therefore, constructing the synthesis pathways to synthesize compatible solutes or other chemicals is another effective approach to combat multiple lignocellulose-derived inhibitors.

The tolerance to malnutrition and heat stress in *Z. mobilis* was significantly improved by integrating three genes (*yfdZ*, *metB*, and *Pfu-sHSP*) into the genome of *Z. mobilis* CP4 (CP4) via a Tn5 transposon (Fig. 2). The genomic integration of the three genes conferred the ability of *Z. mobilis* to grow in a simple chemically defined medium without the need for amino acid supplementation (Zhang et al. 2013). Also, the molecular mechanism of thermotolerance partially overlaps with that of ethanol tolerance based on the result that 60% genes involved in these two mechanisms are shared (Charoensuk et al. 2017). Therefore, stabilization engineering of the membrane structure is a possible strategy to address these two challenges together in the future. Additionally, the salt tolerance of *Z. mobilis* was also improved using a similar approach with the transposon insertion located in *ZMO1122* (*himA*). The resulting mutant strain displayed better fermentation performance under NaCl stress than the wild-type ZM4 strain (Wang et al. 2016). Recently, the regulation of solvent efflux pumps has been shown to be a promising engineering strategy for increasing tolerance to biofuels (Jones et al. 2015). The furfural resistance of *Z. mobilis* was shown to be increased by knockout or downregulation of the expression of the toxic chemical efflux pump–encoding operon, which contains the genes *ZMO0282*, *ZMO0283*, and *ZMO0285*, or by overexpression of the repressor-encoding gene (*ZMO0281*) for this efflux pump–encoding operon (Yang et al. 2015).

Some successful engineering strategies used in other bacteria also show promise for improving the tolerance of *Z. mobilis*. For example, small RNAs (sRNA) have been used as transcriptional regulators to indirectly modulate the activities of sigma factors and elicit tolerance phenotypes. The overexpression of three sRNAs (DsrA, ArcZ, and RprA), which act as activators for the stationary phase sigma factor RpoS, significantly increased the tolerance *E. coli* to carboxylic acid and oxidative stress (Gaida et al. 2013). In addition, other available engineering strategies include the overexpression of GroESL (Zingaro and Papoutsakis 2013), promoter engineering (Alper et al. 2005a), intergenic sequence engineering (Pfleger et al. 2006), lipid engineering (Degreif et al. 2017), and phenotypic engineering using novel artificial transcription factors (Young et al. 2008). These strategies can potentially be developed as novel methods to enhance the tolerance of *Z. mobilis* against various inhibitors.

Cofactor engineering

Cofactors (e.g., NAD(H), NADP(H), and ATP) can supply the energy needed for carbon metabolism and are involved in maintaining redox balance. The results of a previous study showed that the inhibitor acetate can cause a toxic chain reaction by driving carbon flux towards acetate production with excessive NADH accumulation (Yang et al. 2014b). Furthermore, the thermotolerance and salt stress tolerance was related with the level of NADH dehydrogenase encoded by *ndh* gene in respiratory-deficient mutants (Hayashi et al. 2012; Hayashi et al. 2015). Subsequently, a recent study reported that respiratory Ldh is also involved in oxidative and thermal stress resistance in these mutants (Strazdina et al. 2018). To address this problem, different redox cofactor engineering strategies have been successfully implemented to increase the cellular availability of the desired redox cofactor or to alter the cofactor specificity of key enzymes. For example, the co-expression of the genes encoding NADPH-dependent alcohol dehydrogenase and transhydrogenase (*ZMO1771* and *udhA*, respectively), which are responsible for the regeneration of NADPHs, can effectively promote the conversion of furfural and HMF into less toxic corresponding alcohols and increase the ethanol fermentation performance of *Z. mobilis* (Wang et al. 2017) (Fig. 2).

Based on these natural examples of inhibitor-tolerant microbes, it is likely that the comprehensive application of multiple strategies will greatly improve ethanol and inhibitor

tolerance. Combined computational and experimental approaches have been used to improve ethanol tolerance in *E. coli* (Goodarzi et al. 2010). The effect of single-gene perturbations was first experimentally determined, and these data were then used in a computational model to accurately predict the effects of combining multiple perturbations. Furthermore, tolerance engineering can also be combined with some detoxification methods, including neutralization, overliming with calcium hydroxide, activated charcoal, ion exchange resins, and enzymatic detoxification using laccase, which are known for removing various inhibitors from lignocellulosic hydrolysates. The use of such hybrid approaches will be very powerful for the creation of ethanol- and inhibitor-tolerant strains.

Conclusions and outlook

Z. mobilis has been regarded as a model microbe for the production of biofuels and other biochemicals, and the physiological traits of this bacterium under various conditions have been extensively studied. In the past decades, some traditional genetic tools have been developed by various researchers. For example, some reporter genes, such as green fluorescent protein (GFP) and the *Pseudomonas syringae* ice nucleation gene (*inaZ*), have been used as efficient and easily assayable tools to assess promotor activity and are used as "parts" in synthetic biology (Douka et al. 2001; Drainas et al. 1995). Recently, different vectors (Cao et al. 2016; Dong et al. 2011) and recombineering methods, such as RecET (Wu et al. 2017), the FLP-FRT site-specific recombination system (Zou et al. 2012) and CRISPR-Cas9 genome editing technology (Cao et al. 2017a), have also been used in *Z. mobilis* to facilitate the characterization of important target genes and to construct new metabolic pathways. Although various genetic tools are available for *Z. mobilis*, more efficient tools for high-throughput screening of mutants are still needed, such as an adaptive laboratory evolution method based on visualizing evolution in real-time using flow cytometry (Reyes et al. 2012), microfluidics and microfluidic droplet screening systems (Ma et al. 2018), or biosensors based on fluorescence-activated cell sorting (FACS) (Ng et al. 2015).

Although significant progress in tolerance engineering has been made in *Z. mobilis*, the desired strains with complex phenotypes should be continuously developed to overcome multiple inhibitors and/or the simultaneous use of sugars in lignocellulose hydrolysates. The consortia approach that makes use of multiple strains working synergistically, such as mixed cultivation, co-culture, or fed-batch, will possibly enhance lignocellulose-to-ethanol process robustness. Additionally, the direct assimilation of cellulosic or hemicellulosic oligomers based on cell surface engineering is another promising method for future metabolic engineering studies, as these approaches can avoid enzymatic decomposition steps and CCR. Recently, the iterative CRISPR EnAbled Trackable genome Engineering (iCREATE) strategy was used for engineering a strain with complex phenotypes in *E. coli* to meet the requirements of both rapid glucose and xylose co-utilization and hydrolysate inhibitor tolerance, which is another attractive option for eliminating CCR (Liu et al. 2018). In summary, these various approaches will facilitate further metabolic engineering manipulations for the development of more robust *Z. mobilis* strains for the efficient production of ethanol and other biofuels or bio-based chemicals from lignocellulosic biomass.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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