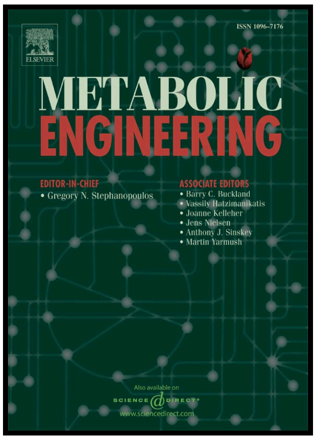

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PII: S1096-7176(18)30036-3
DOI: https://doi.org/10.1016/j.ymben.2018.04.001
Reference: YMBEN1376

To appear in: *Metabolic Engineering*

Received date: 25 January 2018
Revised date: 31 March 2018
Accepted date: 1 April 2018

Cite this article as: Xia Wang, Qiaoning He, Yongfu Yang, Jingwen Wang, Katie Haning, Yun Hu, Bo Wu, Mingxiong He, Yaoping Zhang, Jie Bao, Lydia M. Contreras and Shihui Yang, Advances and Prospects in Metabolic Engineering of *Zymomonas mobilis*, *Metabolic Engineering,* https://doi.org/10.1016/j.ymben.2018.04.001

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Advances and Prospects in Metabolic Engineering of *Zymomonas mobilis*

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DOI: 10.1016/j.ymben.2018.04.001

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DOI: 10.1016/j.ymben.2018.04.001

Abstract:

Biorefinery of biomass-based biofuels and biochemicals by microorganisms is a competitive alternative of traditional petroleum refineries. *Zymomonas mobilis* is a natural ethanologen with many desirable characteristics, which makes it an ideal industrial microbial biocatalyst for commercial production of desirable bioproducts through metabolic engineering. In this review, we summarize the metabolic engineering progress achieved in *Z. mobilis* to expand its substrate and product ranges as well as to enhance its robustness against stressful conditions such as inhibitory compounds within the lignocellulosic hydrolysates and slurries. We also discuss a few metabolic engineering strategies that can be applied in *Z. mobilis* to further develop it as a robust workhorse for economic lignocellulosic bioproducts. In addition, we briefly review the progress of metabolic engineering in *Z. mobilis* related to the classical synthetic biology cycle of "Design-Build-Test-Learn", as well as the progress and potential to develop *Z. mobilis* as a model chassis for biorefinery practices in the synthetic biology era.

Keywords: *Zymomonas mobilis*; Industrial chassis; Biorefinery; Biochemical production; Metabolic engineering; Synthetic biology

DOI: 10.1016/j.ymben.2018.04.001

With the increasing consumption of global fossil resources and the resulting environmental concern, the production of biofuels from alternative and eco-friendly resources has become increasingly important. Lignocellulosic biomass is considered a renewable and sustainable feedstock for bioenergy and biochemical production. Given its naturally favorable physiological attributes, significant efforts have been made to develop *Z. mobilis* as an ideal chassis for biorefinery, especially for the economic production of lignocellulosic biofuels and biochemical **(Fig. 1)**. In this paper, we review the progress and challenges in metabolic engineering of *Z. mobilis* for biotechnology applications. We also review a few recent metabolic engineering strategies that can be applied in *Z. mobilis* to further its development as a model microbe for synthetic biology and biorefinery applications.

Attractive physiological features of *Z. mobilis* for biotechnology

Z. mobilis is a natural facultative anaerobic ethanologenic Gram-negative bacterium with many desirable industrial characteristics (**Table 1**). For example, *Z. mobilis* is generally regarded as safe (GRAS), exhibits very high ethanol tolerance up to 16% (v/v), and can produce ethanol across a broad pH range (3.5-7.5, especially low pH). As a facultative anaerobe, *Z. mobilis* does not require controlled aeration during fermentation, and therefore reducing production costs (Doelle et al., 1993; He et al., 2014; Panesar et al., 2006; Rogers et al., 2007; Yang et al., 2016a).

Z. mobilis uses the efficient Entner-Doudoroff (ED) pathway to anaerobically ferment glucose for ethanol production (Rogers et al., 1982; Swings and De, 1977). Compared to the classical Embden-Meyerhof-Parnas (EMP) pathway for glycolysis in other model species such as *Saccharomyces cerevisiae* and *Escherichia coli*, only 1 mole of ATP is yielded per mole of glucose through the ED pathway (**Table 1**). As previously studied, the ED pathway is less thermodynamically constrained and requires less enzymatic protein than the EMP pathway to sustain the same flux (Flamholz et al., 2013). The highly efficient ED pathway together with pyruvate decarboxylase (Pdc) and two alcohol dehydrogenases (Adh) form the 'backbone' of *Z. mobilis* glycolysis metabolism. Notably, the EMP pathway, pentose phosphate pathway (PPP), and tricarboxylic acid (TCA) cycle are incomplete in *Z. mobilis* since various enzymes within these pathways are not identified in this organism. For instance,

DOI: 10.1016/j.ymben.2018.04.001

phosphofructokinase (Pfk) in the EMP pathway, phosphogluconate dehydrogenase (Pgd) and transaldolase (Tal) in the PPP pathway, as well as 2-oxoglutarate dehydrogenase complex (*sucABCD*) and malate dehydrogenase (Mdh) in the TCA cycle are not present in *Z. mobilis* (Raps and Demoss, 1962; Seo et al., 2005). The deficiency of these enzymes and the incompleteness of the PPP and TCA cycle in *Z. mobilis* thus drive more carbon into the highly efficient glycolysis and ethanol production pathways instead of PPP and/or TCA cycle, which results in ethanol production close to the theoretical maximum level with three- to five-fold lower biomass yield relative to *S. cerevisiae* on a cell basis for *Z. mobilis* (Bai et al., 2008; Lau et al., 2010; Wirawan et al., 2012). In addition, it possesses a high-specific cell surface and consumes glucose faster than *S. cerevisiae* and *E. coli*, leading to higher ethanol productivity (Chen et al., 2017; Ohta et al., 1991; Panesar et al., 2006).

The respiratory chain of *Z. mobilis* displays a uniquely uncoupled energetics and cellular growth with physiology that has been suggested to maintain a low NADH/NAD$^+$ ratio for efficient glycolysis and cellular growth (Hayashi et al., 2015; Kalnenieks et al., 2008; Rutkis et al., 2014; Rutkis et al., 2016). Specifically, *Z. mobilis* has a constitutive respiration chain that uses oxygen as the terminal electron receptor under aerobic conditions, comprising of a type II NADH dehydrogenase (Ndh), coenzyme Q10, and a cytochrome bd terminal oxidase as the major electron carriers, together with some minor or still unidentified constituents (Belauich and Senez, 1965; Sootsuwan et al., 2008). Compared to *E. coli* and *S. cerevisiae* respiration, *Z. mobilis* has a higher rate of oxygen consumption, but yet lower yields of ATP (Agrawal et al., 2017; Rutkis et al., 2014; Rutkis et al., 2016).

Interestingly, a recent work demonstrated that *Z. mobilis* can utilize nitrogen gas as a nitrogen source to produce bioethanol without compromising its ethanol productivity, which could potentially reduce the bioethanol production cost due to the low cost of nitrogen gas as a nitrogen source (Kremer et al., 2015). All these characteristics make *Z. mobilis* an ideal host for industrial biotechnology applications.

Availability of multiple genome sequences for *Z. mobilis* strains

Advances in genome sequencing technologies especially next-generation sequencing (NGS) techniques provide new opportunities to obtain fundamental insights into *Z. mobilis*

DOI: 10.1016/j.ymben.2018.04.001

strains. Since the initial genome sequence of model species ZM4 was published in 2005 and its annotation improved in 2009 (Seo et al., 2005; Yang et al., 2009a), several other strains have been sequenced, including CP4, NCIMB 11163, ATCC 29191, ATCC 29192, ATCC 10988, ATCC 31822, ATCC 31823, NRRL B-12526, and NRRL B-1960 (Chacon-Vargas et al., 2017; Desiniotis et al., 2012; Kouvelis et al., 2011; Kouvelis et al., 2009; Kouvelis et al., 2014; Pappas et al., 2011; Zhao et al., 2012; Zhao et al., 2016) (See list of strains in **Table 2**). Genome comparison using ORFs (open reading frames) with other microorganisms demonstrated that *Z. mobilis* ZM4 has the closest similarity with *Novosphingobium aromaticivorans* (Seo et al., 2005), which is consistent with a previous phylogenetic study based on the 16S ribosomal RNA sequence (Lee et al., 2001). Except for ATCC 31822 and ATCC 31823 containing 26 and 30 scaffolds respectively, all strains have been completely sequenced, and the genome sequences of the first nine strains have been compared with the model strain ZM4 (Yang et al., 2016a).

The genome of NRRL B-1960 strain was sequenced recently, which includes a single 2,045,798-bp circular chromosome and two plasmids with eleven unique genes (Chacon-Vargas et al., 2017). In addition, complete chromosome and plasmid sequences of ZM4 (CP023715) and its engineered xylose-utilizing derivatives 2032 and 8b have been determined and characterized recently. Resequencing of the ZM4 chromosome identified 65 SNPs and a 2,400-bp insertion relative to previously published ZM4 chromosomal sequence (AE008692.2). Four native plasmids were also identified ranging in size from 32 to 39 kb (CP023716-9 for pZM32, pZM33, pZM36, and pZM39 respectively), and harboring in total 150 predicted ORFs (Unpublished data). An evolution tree was built using all protein sequences of each *Z. mobilis* strain through the CVtree3.0 web-server (Zuo and Hao, 2015) to exhibit the phylogenetic relationship among these strains **(Fig. 2)**. The genome sizes of all strains range from 2.01 to 2.22 Mb with two to eight plasmids **(Fig. 2, Table 2)**, which is only half the genome size of *E. coli* (**Table 1**). In addition, the central metabolism of *Z. mobilis* appears to be simpler than that of *E. coli*, appearing to have evolved with a much higher fraction of essential genes and a more rigid metabolism (Widiastuti et al., 2011).

With the availability of various *Z. mobilis* genomes, systems biology studies have provided guidelines for strain improvement (He et al., 2012a; He et al., 2012b; Skerker et al.,

DOI: 10.1016/j.ymben.2018.04.001

2013; Yang et al., 2014a; Yang et al., 2010a; Yang et al., 2014b; Yang et al., 2013; Yang et al., 2009b), and has been reviewed previously (He et al., 2014). In addition, a number of genome-scale metabolic models for *Z. mobilis* have been constructed to provide a deeper understanding of the cellular metabolism, and to pave the road for rational development of this microbial biocatalyst (Lee et al., 2010; Motamedian et al., 2016; Widiastuti et al., 2011).

Engineering *Z. mobilis* strains with expanded substrate utilization

Wild-type *Z. mobilis* can only naturally grow on glucose, fructose, and sucrose as carbon sources, but not on pentoses such as arabinose and xylose, which are abundant sugars within lignocellulosic hydrolysates. To extend its substrate spectrum, different heterologous genes have been selected and transformed into *Z. mobilis* (**Table 3**). For instance, a recombinant *Z. mobilis* CP4 (pZB5) strain has been constructed, which is capable of simultaneous fermentation of pentose and hexose sugars within lignocellulosic hydrolysates into ethanol by introducing two operons encoding xylose assimilation (XylA/B) and PPP enzymes (Tal and Tkt) from *E. coli* (Zhang et al., 1995). Another recombinant *Z. mobilis* CP4 (pZB206) strain has also been engineered to produce ethanol from arabinose sugar by expressing five arabinose metabolism-related genes from *E. coli* (Deanda et al., 1996). Since then, recombinant and evolved *Z. mobilis* strains for pentose sugars utilization have been developed through metabolic engineering and/or adaptation methods (Chou et al., 2015; Dunn and Rao, 2014; Jeon et al., 2005; Ma et al., 2012; Mohagheghi et al., 2002; Mohagheghi et al., 2014; Wang et al., 2016a; Yanase et al., 2012; Zhang et al., 1998).

However, rates of xylose and arabinose consumption in these recombinant strains have been far slower than that of glucose, which significantly prevent these sugars from efficient simultaneous utilization. Consequently, significant efforts have been focused on investigating bottlenecks that prevent xylose and arabinose from efficient utilization in *Z. mobilis*. Our previous study demonstrated that xylose poses more dramatic inhibitory effect, impacting more *Z. mobilis* genes, than major hydrolysate inhibitors such as acetate (Yang et al., 2014a). In addition, the imbalance of $NAD/NADH^+$ during xylose fermentation could reduce overall xylose metabolism efficiency due to the carbon and energy loss while leading to the accumulation of the toxic intermediate xylitol (Feldmann et al., 1992; Kim et al., 2000),

DOI: 10.1016/j.ymben.2018.04.001

which is consistent with studies demonstrating that the deletion of a putative xylose reductase from the chromosome of *Z. mobilis* resulted in decreased xylitol production (Agrawal and Chen, 2011; Agrawal et al., 2012).

In addition to the formation of toxic xylitol, inefficient xylose transport represents another bottleneck. Xylose enters the cell through the promiscuous glucose-facilitated diffusion protein Glf in *Z. mobilis*, which is also involved in arabinose transport and may be blocked by glucose through a competitive inhibition mechanism (Dimarco and Romano, 1985; Dunn and Rao, 2015; Weisser et al., 1996). As a way to address this, expression of a low-affinity xylose transporter XlyE from *E. coli* in *Z. mobilis* has been shown to increase the rate of xylose metabolism at high xylose concentrations (Dunn and Rao, 2014) (**Table 3)**. Besides glucose, xylose, and arabinose, *Z. mobilis* has also been engineered to ferment other minor sugars present in lignocellulosic hydrolysates such as mannose or galactose into ethanol (Weisser et al., 1996; Yanase et al., 1991) (**Table 3)**.

While some potential bottlenecks in xylose and arabinose fermentations by *Z. mobilis* have been identified, ethanol production from lignocellulosic pentose sugars by the engineered *Z. mobilis* strains still lags behind ethanol production from hexose sugars. In order for the industrial ethanol production process to be economical, pentose sugar fermentations by *Z. mobilis* must be improved further especially when lignocellulosic biomass hydrolysates containing toxic compounds such as furfural and acetate are used.

Construction of *Z. mobilis* consolidated bioprocessing (CBP) strains

Consolidated bioprocessing (CBP) refers to the use of a single microorganism to convert lignocellulosic biomass to ethanol through the simultaneous production of cellulolytic and saccharolytic enzymes and the fermentation of released fermentable sugars, which presents a promising technology for cost-competitive biofuel production (Lynd et al., 2005). To directly convert cellulose, *Z. mobilis* needs to be capable of secreting at least three categories of plant cell wall degrading enzymes: endoglucanase (EC3.2.1.4), exoglucanase (EC3.2.1.91), and β-glucosidase (EC3.2.1.21). Except that the enzymatic activity of β-1,4-endoglucanase (encoded by ZMO1086) was determined by a previous study (Rajnish et al., 2008), *Z. mobilis* lacks of most cellulolytic enzymes.

DOI: 10.1016/j.ymben.2018.04.001

Different cellulolytic enzymes from other species have thus been expressed in *Z. mobilis*, and the resulting recombinant strains have been demonstrated to be able to ferment cellulosic polysaccharide directly into ethanol **(Table 3)**. For example, *Z. mobilis* has been engineered to ferment cellobiose with a yield of 0.49 g ethanol/g substrate by expressing a heterologous β-glucosidase from *Ruminococcus albus* with a native signal peptide (Yanase et al., 2005). Heterologous endo-1,4-β-D-glucanases, such as two enzymes of E1 and GH12 from *Acidothermus cellulolyticus* or endo-1,4-β-D-glucanase from *Enterobacter cloacae*, have also been successfully expressed in *Z. mobilis* (Linger et al., 2010; Thirumalai Vasan et al., 2011). Subsequently, a heterologous β-D-glucosidase from *Bacillus polymyxa* has been expressed in *Z. mobilis*, where its secretion was facilitated by the signal peptide of ZMO1086 (Luo and Bao, 2015). More recently, all cellulolytic enzyme encoding genes for endoglucanase, exoglucanase, and β-glucosidase from *Trichoderma reesei* have been transformed into *Z. mobilis*, making the recombinant strain capable of directly utilizing pretreated lignocellulosic feedstocks as fermentation substrates (Venkatesh, 2015) (**Table 3**).

Although these results suggest that *Z. mobilis* can be a promising CBP platform microorganism with the capacity to express and secret cellulolytic enzymes through metabolic engineering, other issues need to be resolved to make *Z. mobilis* as an ideal CBP platform. For example, the expression and secretion of heterologous cellulases are always energetically costly, especially for the overexpression of multiple heterologous enzymes simultaneously, which inevitably compete with the cell growth and ethanol production. It could be a challenge to develop *Z. mobilis* CBP strains for commercial biofuel production considering that only 1 mole of ATP is produced per mole of glucose. Therefore, metabolic engineering efforts in *Z. mobilis* should be focused primarily on converting all pentose and hexose sugars into bioproducts of biofuels and/or high-valued biochemicals efficiently instead (Yang et al., 2016a).

Engineering *Z. mobilis* for a variety of biochemical products

Other than ethanol, *Z. mobilis* possesses endogenous metabolic pathways to produce other metabolic byproducts such as sorbitol, levan, glycerol, as well as lactic, gluconic, succinic, and acetic acids (Barrow et al., 1984; Dawes et al., 1966; Kim et al., 2006; Viikari

DOI: 10.1016/j.ymben.2018.04.001

and Gisler, 1986; Wecker and Zall, 1987), as discussed in detail in other reviews (He et al., 2014; Rogers et al., 2007).

The potential utility of *Z. mobilis* for the commercial production of other biochemicals would be greatly enhanced by the introduction and efficient expression of other heterologous pathways through metabolic engineering **(Table 3)**. For example, *alaD* gene encoding L-alanine dehydrogenase from *Bacillus sphaericus* has been cloned and introduced into *Z. mobilis*, leading to the production and excretion of 7.5 g/L alanine by this recombinant strain (Uhlenbusch et al., 1991). Likewise, a recombinant *Z. mobilis* strain with imported D-lactate dehydrogenase genes from *Leuconostoc* sp. into different genome locations has been demonstrated to produce D-lactate at yields higher than 99.7% at pH 5.0 (Kim et al., 2014). Similarly, the polyhydroxybutyrate (PHB) operon *phbCAB* from *Ralstonia eutropha* has been engineered into *Z. mobilis* and enzymatic activities of PhbA (β-ketothiolase) and PhbB (acetoacetyl-CoA reductase) have been detected with PHB accumulation (Lai, 2006). In a different study, the expression of four carotenoid biosynthetic genes (*crtB*, *crtE*, *crtI*, *crtY*) in *Z. mobilis* from *Erwinia uredovora* resulted in the production of β-carotene (Misawa et al., 1991).

In addition, an engineered *Z. mobilis* ZM4 strain has also been constructed for isobutanol production through the introduction of the 2-ketoisovalerate decarboxylase gene (*kivd*) and alcohol dehydrogenase gene (*adhA*) from *Lactococcus lactis* (He et al., 2014). More recently, heterologous pathways to divert pyruvate into 2,3-butanediol (2,3-BDO) production from ethanol have been explored in *Z. mobilis* (Yang et al., 2016b). The engineered strain containing three heterologous genes encoding acetolactate synthase (*als*), acetolactate decarboxylase (*aldC*), and butanediol dehydrogenase (*bdh*) has been shown to exhibit high 2,3-BDO production at a titer of greater than 10 g/L in batch fermentations (Yang et al., 2016b). Furthermore, the possibility of engineering *Z. mobilis* for farnesene production has also been suggested through the insertion of a farnesene synthase gene into *Z. mobilis* (Yang et al., 2016a). All these studies showed that *Z. mobilis* has great potential for the production of an extended repertoire of value-added biochemicals. However, the resulting titers of these biochemicals are still low compared to that of endogenous ethanol, which may be due to the essentiality of PDC and ADH for glycolysis in *Z. mobilis* to provide energy and intermediates

DOI: 10.1016/j.ymben.2018.04.001

for cellular growth. Although a few attempts have been carried out to delete *pdc* gene, there is no stable *pdc* mutant confirmed yet. It is crucial to manipulate the *pdc* and *adh* genes to divert carbon flux from ethanol production to the new targeted biochemicals while keeping redox balance, and a significant amount of metabolic engineering work still needs to be done to increase the titer, rate, and yield of these bioproducts for future commercialization applications .

Metabolic engineering strategies to optimize energy metabolism in *Z. mobilis*

Quite a few innovative metabolic engineering strategies have been developed for energy and redox balance recently such as the introduction of non-natural cofactors, coproduction of value-added byproducts for redox balance, and the combination of pathway engineering and genome editing, which can be applied in *Z. mobilis* to establish a vigorous cellular metabolism and efficient biochemical production.

For example, the strategy of co-production of 1,3-propanediol (PDO) and lactate from glycerol was used in *Klebsiella oxytoca* to achieve redox balance and therefore high-level production of both PDO (76.2 g/L) and optically pure D-lactate (111.9 g/L) by re-arranging the coordination between the glycerol oxidative and reductive branches (Xin et al., 2017). Another strategy to optimize energy metabolism is the introduction of non-natural cofactors such as nicotinamide cytosine dinucleotide (NCD) as energy carriers operating orthogonal to the native energy transfer systems (Wang et al., 2017c). Furthermore, heterologous pathway engineering and genome editing strategies were combined to develop *E. coli* strain EB243 in which 33 native genes were deleted and 5 heterologous genes introduced. This subsequent engineered strain produced 20 g/L butanol with a yield of 34% (w/w, 83% of theoretical yield) in batch fermentation without the supplementation of any antibiotics or inducers (Dong et al., 2017). Taken together, metabolic engineering strategies like these for energy and redox balance provide guidance for future metabolic engineering efforts in *Z. mobilis* considering the low ATP production and few energy production options in *Z. mobilis*.

Engineering *Z. mobilis* for improved robustness

Besides the toxic end-products such as ethanol produced during fermentation, other

DOI: 10.1016/j.ymben.2018.04.001

inhibitory compounds could be generated and present in the lignocellulosic hydrolysates or slurries during the processes of pretreatment and enzymatic hydrolysis to release mono-sugars from recalcitrant lignocellulosic biomass. These inhibitory compounds include furans such as furfural and hydroxymethylfurfural (HMF), weak acids such as formic acid, levulinic acids, and acetic acid, as well as phenolic aldehydes and inorganic salts. As such, these inhibitors are considered one of the key barriers in value-added chemical production from biomass. These inhibitors have negative effects on *Z. mobilis* cellular growth and metabolism as well as on the production of desired bioproducts (Dong et al., 2013; Franden et al., 2013; Skerker et al., 2013; Yi et al., 2015), making characterizing and engineering tolerance an important effort.

With different genome projects of *Z. mobilis* performed as discussed above **(Fig. 2, Table 2)** and the rapid progress in NGS and mass spectrometry technologies, systems biology approaches have been widely applied to identify genetic elements that correlate with desired phenotypes. These approaches include comparative genomics, transcriptomics, proteomics, metabolomics, and high-throughput (HTP) genetics which provide guidance for improving *Z. mobilis* strain robustness **(Table 4)**. For instance, many microarray-based transcriptomic studies have been carried out for different *Z. mobilis* strains grown under different stressful conditions such as high oxygen level (2.5 L/min), high glucose concentration (220 g/L), and the supplementation of ethanol (5% or 6% v/v) or hydrolysate inhibitors (i.e., furfural, acetate, and phenolic aldehydes). Based on these studies, candidate genes, sRNAs, and regulatory 5′ UTRs (untranslated regions) correlated to inhibitor tolerance improvement in *Z. mobilis* have been identified and characterized (Cho et al., 2017; Cho et al., 2014; He et al., 2012a; He et al., 2012b; Skerker et al., 2013; Yang et al., 2014a; Yang et al., 2010a; Yang et al., 2014b; Yang et al., 2013; Yang et al., 2009b; Yi et al., 2015; Zhang et al., 2015b). Recent examples include the identification of regulatory 5′ UTRs that are naturally responsive to ethanol and other inhibitors for post-transcriptional gene regulation (Cho et al., 2017). Systems biology studies have also led to the identification of a RNA-binding protein Hfq that is involved in conferring tolerance to multiple hydrolysate inhibitors such as acetate, vanillin, furfural, and HMF in *Z. mobilis* (Yang et al., 2010b; Yang et al., 2009b) **(Table 4)**.

Previous studies have observed that, during growth, *Z. mobilis* is capable of converting

DOI: 10.1016/j.ymben.2018.04.001

the aldehydes furfural, HMF, vanillin, 4-hydroxybenzaldehyde, and syringaldehyde to their alcohol forms of furfuryl, 5-hydroxymethylfurfuryl, vanillyl, 4-hydroxybenzyl, and syringyl alcohol respectively (Franden et al., 2013; Gu et al., 2015; Yi et al., 2015). This observation suggested that *Z. mobilis* might harbor the native oxidoreductases to catalyze theses toxic aldehydes into benign alcohol-form compounds. Consistent with this assumption, several reductases encoding genes responsible for the conversion of phenolic aldehydes (ZMO1696 and ZMO1885) or furan aldehydes (ZMO1771) were identified and the overexpression of these genes resulted in enhanced inhibitor tolerance to phenolic aldehyde and furan aldehydes, respectively (Wang et al., 2017d; Yi et al., 2015) **(Table 4)**. More recently, a transcriptomic study indicated that cellulose production and flagella activity are likely to facilitate the stable flocculent behavior in ZM401, which renders improved tolerance to inhibitory compounds of this mutant strain compared to the non-flocculating wild-type strain *Z. mobilis* ZM4 (Jeon et al., 2012; Zhao et al., 2014).

In addition, genome-resequencing analysis has also been applied to identify the underlying genetic changes responsive for the altered phenotypes in robust *Z. mobilis* mutants generated through mutagenesis or adaption (Charoensuk et al., 2017; Dunn and Rao, 2015; Joachimsthal et al., 1998; Liu et al., 2017; Mohagheghi et al., 2014; Mohagheghi et al., 2015; Wang et al., 2016b). For example, a 1.5-kb deletion in the acetate-tolerant strain AcR was identified to contribute to *nhaA* (encoding sodium proton antiporter) over-expression and the consequential enhanced sodium acetate tolerance capability through this strategy (Yang et al., 2010a), which is consistent with a recent similar result in another acetate-tolerant mutant ZMA-167 (Liu et al., 2017) **(Table 4)**. Continuous culture methodology has also been used to evolve and adapt *Z. mobilis* 8b strain for improved ethanol productivity using corn stover hydrolysate. The resulting SS3 mutant exhibited a higher ethanol yield of 75.5% compared to the 64% ethanol yield of 8b. Two genes (ZMO0153 and ZMO0776) significantly down-regulated in SS3 in both rich medium and hydrolysate conditions might be involved in the improved hydrolysate tolerance of the SS3 strain (Mohagheghi et al., 2015).

It is now generally accepted that most cellular phenotypes are affected by multiple genes that are widely distributed throughout the genome. Therefore, deletion or over-expression of a single gene can hardly reach an optimal global phenotype due to the complexity of metabolic

DOI: 10.1016/j.ymben.2018.04.001

landscapes. Development of the global transcriptional machinery engineering (gTME) therefore has attracted much attention in the field of strain engineering as a powerful approach to address the problem of simultaneously affecting the expression of multiple genes (Alper and Stephanopoulos, 2007). gTME is a method of directed evolution in which sigma factors and other components of the cellular transcription machinery are subjected to several rounds of mutation via error-prone PCR or DNA shuffling. Mutations of these elements alter the efficiency and promoter preferences of RNA polymerase, modulating the transcriptome at a global level (Gardella et al., 1989; Owens et al., 1998), and has been successfully applied to elicit new cellular phenotypes, resulting in improved stress tolerance, enhanced substrate utilization and metabolite production in a variety of organisms. By constructing and screening random mutagenesis libraries of the global transcription sigma factor RpoD (σ^{70}) obtained through error-prone PCR, tolerance to furfural and ethanol has been greatly improved in *Z. mobilis* (Tan et al., 2016; Tan et al., 2015). This approach could be effective for improving other similar complex phenotypes that involve multiple genes of *Z. mobilis* while providing guidance for the assembly of synthetic transcriptional machinery.

Z. mobilis **genes employed in metabolic engineering of other microorganisms**

The knowledge gained from the model ethanologen *Z. mobilis* can be extended and used directly in metabolic engineering of other microbial biocatalysts. A few genes from *Z. mobilis* have already been deposited into the standard biology of the International Genetically Engineered Machine (iGEM) registry, especially those with unique characteristics. These include genes encoding for pyruvate decarboxylase Pdc (ZMO1360, EC 4.1.1.1), alcohol dehydrogenase AdhB (ZMO1596, EC1.1.1.1), extracellular sucrase SacC (ZMO0375, EC 3.2.1.26), and glucose-facilitated diffusion protein Glf (ZMO0366).

These genes, in particular those encoding for the ethanol production (*pdc* and *adh*) in *Z. mobilis,* have been introduced into other microorganisms for efficient ethanol production. For example, an engineered *E. coli* strain is able to produce acetaldehyde at a titer of 0.73 g/L from glucose by the introduction of Pdc from *Z. mobilis* and NADH oxidase (Nox) from *L. lactis* (Balagurunathan et al., 2017). The fusion enzyme of Pdc and AdhB from *Z. mobilis* (Part:BBa_K1122673), generated by fusing C terminus of Pdc to N terminus of AdhB, also

DOI: 10.1016/j.ymben.2018.04.001

increased the ethanol yields and productivities in *E. coli* and lactic acid bacteria (Hong et al., 2010; Lewicka et al., 2014; Nichols et al., 2003; Piriya et al., 2012). Similarly, the introduction of *Z. mobilis* Pdc and AdhB into the acetolactate synthase (Als)-deficient mutant of *Klebsiella pneumoniae* has resulted in a *K. pneumoniae* strain that can convert pyruvate into ethanol. Moreover, the heterologous expression of *pdc* and *adhB* genes recovers glycerol metabolism in the Als-negative mutant of *K. pneumoniae*, enabling the production of PDO by the engineered strain to increase from 9.05 to 14.26 g/L by preventing pyruvate accumulation (Lee et al., 2014a).

The glucose-fructose oxidoreductase (Gfor) from *Z. mobilis* has also been expressed in *E. coli* for lactobionic acid (LBA) production from whey-derived lactose (Goderska et al., 2015). Likewise, the L-asparaginase encoding gene from *Z. mobilis* has been expressed extracellularly and intracellularly (cytoplasmically) in *E. coli*, yielding 0.13 IU/mL extracellular L-asparaginase and 3.6 IU/mL intracellular L-asparaginase after 4 h of IPTG (isopropyl β-D-1-thiogalactopyranoside) induction (Einsfeldt et al., 2016).

In addition to the utilization of native *Z. mobilis* genes, fundamental understanding of the underlying mechanisms for inhibitor tolerance gained in *Z. mobilis* studies can also be extended to help improve the robustness of other microorganisms. For example, our previous studies indicated that the overexpression of sodium proton antiporter NhaA or global regulator Hfq in *Z. mobilis* contribute to its enhanced inhibitor tolerance, and the identification and overexpression of their corresponding homologous genes in *S. cerevisiae* have been proven to help improve the robustness of *S. cerevisiae* toward inhibitors like sodium acetate (Yang et al., 2010a; Yang et al., 2010b).

Metabolic engineering in the synthetic biology era

As an interdisciplinary branch of biology and engineering, synthetic biology has achieved many goals toward rational design of biological systems to meet the needs of biotechnological applications. Here, we review metabolic engineering progress and prospects of *Z. mobilis* related to the classical synthetic biology cycle of “Design-Build-Test-Learn”.

Selection and characterization of biological parts for metabolic engineering in *Z.*

DOI: 10.1016/j.ymben.2018.04.001

mobilis

The goal of synthetic biology is to extend or modify the behavior of organisms and engineer them to perform new tasks. Design of new behavior is implemented with the fundamental components or 'parts', including DNA, RNA, and proteins (Andrianantoandro et al., 2006). The robust modification and characterization of these individual parts mean that they can be combined to produce new pathways and devices that give a predictable response, such as the biosynthesis of a desired bioproduct to a specified level under defined growth conditions in the chassis cell (**Fig. 3**).

DNA biological parts (promoters, terminators, RBSs, and TIGRs): A functional gene consists of a promoter, a ribosome binding site (RBS) sequence, the protein coding ORF, and a terminator, while between adjacent genes or within a operon, it further includes a tunable intergenic region (TIGR) (Ellis et al., 2011; Pfleger et al., 2006). Promoters and terminators are stretches of DNA upstream and downstream of genes respectively, that control both the frequency at which the gene is transcribed and the rate at which mRNA is degraded (Deaner and Alper, 2016). The use of promoter libraries has enabled the exploration of a greater dynamic range of gene expression (Alper et al., 2005) and of various combinatorial strategies for regulation of multiple genes. Some multi-gene regulation strategies that have been reported include the use of different inducible promoters for each gene or the use of the same inducible promoter for each gene but with varying promoter strength (Nielsen and Keasling, 2016). Likewise, translation efficiency in terms of translation initiation rates can be controlled by designing RBS with different strengths (Na and Lee, 2010; Salis et al., 2009), which has been applied in biosynthetic pathway engineering to help increase the production of astaxanthin (Zelcbuch et al., 2013) and fatty acids (Xu et al., 2013) in *E. coli*.

DNA parts are the primary biological parts that have being investigated in *Z. mobilis*. For example, P_{lac}, P_{tac}, P_{T7}, P_{bad}, and P_{tet} from *E. coli* have been incorporated as inducible promoters for heterologous gene expression in this organism (Arfman et al., 1992; Byun et al., 1986; Carey et al., 1983; Skerker et al., 2013; Yang et al., 2016b; Zeng et al., 2010). Since glycolytic enzymes of *Z. mobilis* represent approximately half of the total cytoplasmic proteins (Algar and Scopes, 1985), and glycolytic genes are expressed at roughly 100-fold higher levels than most housekeeping genes, promoters of many glycolytic genes have been

DOI: 10.1016/j.ymben.2018.04.001

sequenced to establish common molecular features that may specify their efficient transcription (Barnell et al., 1990; Conway et al., 1991; Conway and Ingram, 1988; Conway et al., 1987b; Conway et al., 1987c). The -35 and -10 regions of *Z. mobilis* promoters are not identical to standard promoters of *E. coli*, but a preliminary promoter consensus sequence of glycolytic genes has been established, which could partly explain the insufficient expression of *E. coli* genes in *Z. mobilis* (Conway et al., 1991; Pond et al., 1989; Sprenger et al., 1993). Although the promoter transferability between *E. coli* and *Z. mobilis* is lacking, the understanding of the natural expression of genes within the *Z. mobilis* glycolytic pathway have allowed the use of strong and constitutive promoters within this pathway (especially P_{gap}, P_{eno}, P_{pdc}, and P_{adh}) for the expression of heterologous genes in *Z. mobilis* (Mackenzie et al., 1989; Reynen et al., 1990; Zhang et al., 1995).

Nevertheless, studies to date have primarily relied on the use of limited strong and constitutive promoters for expression of heterologous or homologous pathway genes in *Z. mobilis*. Controllable gene expression within the engineered strains for well-balanced carbon and energy metabolism in different growth phases and environmental conditions should be considered, and strategies to fulfill this goal should be developed, including systematic identification and characterization of inducible promoters, RBSs, and TIGRs elements in *Z. mobilis* or those elements that have been studied in other model bacteria.

Regulatory RNAs (sRNA, UTRs, and CRISPR RNAs): In recent years, regulatory noncoding RNAs, which include riboswitches, 5′ and 3′ UTRs, *cis*-acting antisense RNAs, and *trans*-acting RNAs, have been increasingly recognized and utilized as key modulators for gene expression optimization to precisely and predictably control metabolic flux (Chae et al., 2017; Tong et al., 2015; Villa et al., 2017). By using mRNAs as targets, RNA regulators can affect transcription, translation, as well as RNA stability, maturation, and processing (Romby and Charpentier, 2010; Vazquez-Anderson and Contreras, 2013; Waters and Storz, 2009).

Among the regulatory RNAs, riboswitches represent a class of sensors that exhibit structural changes to regulate transcription or translation of mRNAs by sensing small molecule metabolites, which are elements present in the 5' UTRs of mRNA molecules that typically bind to ligands and regulate the expression of downstream genes (Mellin et al., 2014). These switches can regulate the expression of noncoding RNAs and control protein

DOI: 10.1016/j.ymben.2018.04.001

expression (Chen and Gottesman, 2014), and have been used to construct biosensors for biotechnology applications (Mellin and Cossart, 2015; Zhang et al., 2015a). In a similar way, regulatory small RNAs (sRNAs) have emerged as key regulators of gene expression in bacteria. sRNAs in prokaryotes are relatively short (50 to 300 nucleotides) and not translated. These sRNAs represent a subset of noncoding RNAs that can be both activators and repressors for regulating proteins and mRNAs via a variety of mechanisms (Kang et al., 2014; Livny and Waldor, 2007; Wassarman, 2002). In most cases, sRNA regulatory function depends on base pairings between the sRNA and its target mRNA. The base paring of sRNA-mRNA primarily occurs in the 5′ UTRs of mRNAs and most often involves in RBS (Repoila and Darfeuille, 2009). Although not as applied in the context of metabolic engineering yet, another potential tool for engineering metabolic fluxes include both 5' UTRs and 3' UTRs, typically used to regulate gene expression level in response to various metabolites or environmental conditions (Gossringer and Hartmann, 2012; Nechooshtan et al., 2009; Oliva et al., 2015; Toledo-Arana et al., 2009). All these strategies have been discussed in detail in other articles (Chae et al., 2017; Tong et al., 2015; Vazquez-Anderson and Contreras, 2013; Villa et al., 2017)

A few engineering efforts that have involved the use of regulatory RNAs in bacterial organisms include: **1**) The application of riboswitch regulation in vitamin B12 production in *Bacillus megaterium* (Moore et al., 2014); **2)** The design and use of synthetic sRNAs for substantial increase of cadaverine production in *E. coli* (Na et al., 2013); **3**) Optimization of expression levels of multiple natural sRNAs in *E. coli* to improve acid tolerance (Gaida et al., 2013); and **4**) The use of recently developed small transcription activating RNAs regulators (STARs) to disrupt the formation of an intrinsic transcription terminator placed upstream of a gene in *E. coli* that precisely control gene expression (Chappell et al., 2015).

Early in 2006, three riboswitches (two cobalamin and one TPP riboswitches) were predicted in *Z. mobilis* genome by computational analysis using CMfinder. Even though they had not been experimentally confirmed, this study suggested that regulatory elements were likely present in this organism (Cho et al., 2017; Yao et al., 2006). Regulatory RNAs such as sRNAs and 5' UTRs have also been investigated recently in *Z. mobilis* by transcriptomic analysis and computational predictions (Cho et al., 2017; Cho et al., 2014). Fifteen novel

DOI: 10.1016/j.ymben.2018.04.001

sRNAs were identified, including three (Zms2, Zms4, and Zms6) that are naturally differentially expressed under aerobic and anaerobic conditions and three (Zms2, Zms4, and Zms18) that are differentially expressed under 5% (v/v) ethanol-supplemented media. This study thus suggests that sRNAs may play a regulatory role in ethanol production or stress tolerance in *Z. mobilis* (Cho et al., 2014). Among 36 regulatory 5′ UTRs candidates studied under ethanol (5% or 6%, v/v), acetate (10 g/L), and xylose (10 g/L), UTR_ZMO0347 (5′ UTR of gene ZMO0347 encoding the RNA binding protein Hfq) was found to down-regulate downstream gene expression under ethanol stress (Cho et al., 2017). This is the first time that functional native 5′ UTRs have been experimentally confirmed and characterized in *Z. mobilis*. The regulatory role of these 5′ UTRs in *Z. mobilis* provide new strategies for engineering robust industrial strains as well as novel responsive regulatory biological parts for controllable gene expression in this organism (Cho et al., 2015; Leavitt and Alper, 2015; McKeague et al., 2016; Qi and Arkin, 2014; Sowa et al., 2015). The discovery of sRNAs and regulatory UTRs could be impactful to metabolic engineering efforts as these regulators have been implicated in ethanol stress response.

More recently, CRISPR (clustered regularly interspaced short palindromic repeat) RNAs have emerged as a unique family of regulatory RNAs (Sorek et al., 2008), which share certain similarities with eukaryotic siRNA (short interfering RNA). This relatively new area has presented an exciting new research arena for metabolic engineering. Given the wide interest in these systems, native CRISPR systems have also been investigated in *Z. mobilis* and current results suggested that this organism possesses a type I-F CRISPR-Cas (CRISPR associated) system that is actively expressed during immune interference under normal growth conditions (Dong et al., 2016). In addition, a type II CRISPR-Cas9 system of *Streptococcus pyogenes* has been introduced and reconstructed in *Z. mobilis,* whereby small guide RNAs can direct the Cas9 nuclease to knockout the replicase genes of native *Z. mobilis* plasmids (Cao et al., 2017). These studies demonstrate that powerful CRISPR techniques (endogenous or exogenous) may be used for genome engineering efforts in *Z. mobilis*.

Although an increasing number of regulatory RNAs have been identified and characterized in *Z. mobilis*, significant efforts are still needed to characterize and expand regulatory RNAs and to develop efficient genome editing tools for effective pathway design

DOI: 10.1016/j.ymben.2018.04.001

and engineering.

Protein engineering through rational design and directed evolution: Biological components can be expanded by directly redesigning proteins, particularly altering enzymes for enhanced activity, substrate or product specificity, and modified regulation (Foo et al., 2012; Marcheschi et al., 2013). The enzyme activity can be improved by using traditional error-prone PCR libraries, site-directed and site-saturation mutagenesis, DNA shuffling and cross-extension techniques to alter the sequence of existing proteins and to change the substrate binding pocket (Edelheit et al., 2009; Labrou, 2010; Leonard et al., 2010; Werkman et al., 2011). Computational protein design tools, which can identify core parts within protein structures as engineering targets and even allow *de novo* protein design from scratch, have proven highly useful in enzyme engineering efforts (Davey and Chica, 2012). For instance, formolase (FLS) was designed by computational tools, solving the difficulty of introducing heterologous carbon fixation cycles into *E. coli* (Siegel et al., 2015).

In addition, protein engineering strategies to engineer the active site and pocket of an enzyme can also facilitate metabolic engineering advancement by tailoring the biocatalysts to efficiently convert non-native substrates and/or to produce non-native compounds with high specificity for high yields (Zhang et al., 2010; Zhang et al., 2008). For example, the catalytic activity of a diol dehydratase in *K. oxytoca* toward a non-native C4 triol of 1,2,4-butanetriol was enhanced after rational engineering, which led to the novel 1,4-butanediol biosynthesis pathway from xylose (Wang et al., 2017b). Furthermore, regulatory elements (e.g. environmentally-responsive elements) at the protein level can also be engineered, and this strategy has been applied to improve the production of glucosamine (Deng et al., 2005) and L-phenylalanine (Liu et al., 2014a) in *E. coli*.

Protein directed evolution has been applied to identify mutants with modified enzyme productivity, substrate specificity, cofactor requirements, or protein stability. Moreover, it can also help alleviate rate-limiting steps by enhancing the catalytic rate of enzymes (Abatemarco et al., 2013). This specific strategy has led to the significant improvement of xylose fermentation performance in *S. cerevisiae* with a yield of 0.45 g ethanol/g xylose (Lee et al., 2014b). In addition, *S. cerevisiae* was engineered for growth on arabinose as a sole carbon

DOI: 10.1016/j.ymben.2018.04.001

source in minimal medium with growth rates upwards of 0.05 h^{-1} by directed evolution and pathway engineering of an alternative arabinose catabolic pathway (Lee et al., 2016). Similarly, the isoprene production in *S. cerevisiae* reached 640 mg/L and 3.7 g/L in aerobic batch and fed-batch fermentations respectively after introducing an engineered isoprene synthase obtained through directed evolution into the *GAL4*-overexpressing strain (Wang et al., 2017a).

Protein engineering strategies have also been reported in *Z. mobilis*. However, they were mainly focused on three enzymes: pyruvate decarboxylase Pdc, alcohol dehydrogenase AdhB, and levansucrase. Protein design by site-directed mutagenesis on Pdc has resulted in mutants with changes in specific activity, cofactor (ThDP or Mg^{2+}) binding, as well as catalytic activity and stability (Bruhn et al., 1995; Candy and Duggleby, 1998; Chang et al., 1999; Diefenbach et al., 1992; Pohl, 1997). Levansucrase has also been subjected to site-directed mutagenesis and the enzyme activity and transfructosylation products were affected (Li et al., 2011; Li et al., 2008). Moreover, random mutagenesis was performed on AdhB and variant forms were generated with increased thermostability and altered substrate specificity (Rellos et al., 1997; Rellos et al., 1998; Rellos et al., 1995; Rellos and Scopes, 1994). Even with these reported works in *Z. mobilis*, efforts should still be focused more on protein engineering through rational design and directed evolution strategies to develop efficient and effective enzymes for future metabolic pathway engineering in this organism.

Verification of biological parts: Different reporter genes such as β-galactosidase (LacZ), ice nucleation activity, and several fluorescent protein genes have been explored for analysis of gene transcription and promoter function in *Z. mobilis*. As an example, general promoter structure has been characterized in *Z. mobilis* using LacZ as a reporter (Conway et al., 1987a). This study demonstrated that *Z. mobilis* promoter sequences share several features with *E. coli* ones such as similar consensus sequences in the -10 region and partial sequence homology in the -35 region. The sequence AGGA was also identified as a potential RBS in *Z. mobilis*, appearing 8-12 base pairs upstream from the start codon (Conway et al., 1987a). Using LacZ as a reporter, native promoters for *adhB* and *gap* (encoding glyceraldehyde-3-phosphate dehydrogenase) were identified later in *Z. mobilis* (Burchhardt et

DOI: 10.1016/j.ymben.2018.04.001

al., 1993; Mackenzie et al., 1989). An ice nucleation gene *inaZ* cloned from *Pseudomonas syringae* was also expressed in *Z. mobilis* as a sensitive reporter, and used to characterize the native P*pdc* promoter (Drainas et al., 1995).

Green fluorescent protein (GFP) is a unique marker that can be identified by noninvasive methods. It has been confirmed useful as a reporter for gene expression in *Z. mobilis* (Douka et al., 2001). In addition to promoter identification, a GFP reporter system has also been used to characterize 5′ UTR candidates for their abilities to regulate downstream gene expression. Using this *in vivo* fluorescence-based screening system, one UTR (UTR_ZMO0347) was confirmed to respond to ethanol stress in *Z. mobilis* (Cho et al., 2017). Other fluorescent proteins such as yellow fluorescent protein (YFP) and mCherry have also been employed in *Z. mobilis* as the reporter to analyze promoter strengths (Dunn and Rao, 2015; Flamholz et al., 2013). Using transcriptional fusions to the *venus* and *mCherry* fluorescent protein genes, eight *pdc* promoter variants with varying strengths were identified from a library of *pdc* promoter mutants constructed by error-prone PCR (Flamholz et al., 2013).

Biological parts assembly

Various native plasmids, broad host-range vectors or shuttle vectors, gene transfer and expression systems have been constructed and used in *Z. mobilis*. Additionally, a series of genetic tools have been explored and are now routinely used for metabolic engineering in *Z. mobilis*, including transformation methods, such as conjugation and electroporation, and transposon mutagenesis strategies (Cho et al., 2017; Dong et al., 2011; He et al., 2014; Panesar et al., 2006; Skerker et al., 2013; Sprenger et al., 1993). All these genetic toolkits now make metabolic engineering in *Z. mobilis* feasible and practical. In addition, investigations of DNA restriction-modification (R-M) systems in *Z. mobilis* helped reveal that transformation efficiency can be improved with the addition of a restriction inhibitor for more amenable strain development (Kerr et al., 2011; Wu et al., 2013).

A number of DNA assembly methods have been developed, which can be divided into two categories: homology-based methods and restriction-based methods such as Gibson assembly (Gibson et al., 2008), BioBrick (Shetty et al., 2008), and Golden Gate (Engler and Marillonnet, 2014). By changing reaction protocols and linker regions between DNA parts to

DOI: 10.1016/j.ymben.2018.04.001

be assembled, these new methods have improved efficiency, fidelity, and modularity, leading to simple design and bench-side operations (Chao et al., 2015). The strengths and limitations of each method have been reviewed previously (Chao et al., 2015; Liu et al., 2015) and will not be discussed in detail here. Gibson assembly is a homology-based approach and has being successfully applied in *Z. mobilis* to develop an *hfq* mutant strain (Cho et al., 2017). Despite the usefulness of homology-based approaches, restriction-based methods are often employed. BioBrick strategy allows modular assembly of parts in a way that the restriction sites are retained in the 5′ and 3′ termini of the final product, but lost in between the assembled parts. As a result, restriction sites can be recycled and larger products can be assembled (Shetty et al., 2008). A 3.0-kb BioBrick compatible minimized shuttle vector (pEZ15Asp) has been designed and synthesized for efficient pathway construction in *Z. mobilis*. This shuttle vector contains only the essential elements: replication origins for both *E. coli* and *Z. mobilis*, an antibiotic marker of the spectinomycin resistance gene *addA*, and multiple cloning sites with BioBrick adapters (Yang et al., 2016b). As yet a different approach, Golden Gate assembly can generate user defined overhangs by simultaneous assembling of multiple fragments in a defined order. With this method, 5′ UTRs along with the first 90-bps of the downstream mRNA in *Z. mobilis* were cloned for 36 candidates right in front of the *gfp* gene in frame for the construction of GFP-reporter plasmids with 5′ UTR (Cho et al., 2017). Initial implementations of these assembly methods have been useful in *Z. mobilis* and should continue to be used in the future.

Rapid computational development has empowered modularization of genetic elements and computer-aided DNA assembly. Modularization of genetic elements can enable the convenient construction and optimization of metabolic networks with complicated structure from heterologous elements. Promoter modularization studies of genetic elements have been constructed by Biobrick, allowing the potential of designing and easily constructing various metabolic and regulatory pathways with simple combinations of related DNA parts (Qi et al., 2015; Shetty et al., 2008). DNA assembly software such as J5 DNA, AtuoBio CAD, R2oDNA designer, SBROME, and genome editing suite BioStudio can help facilitate the creation and screening of genomic libraries and the design of synthetic gene modules (Qi et al., 2015). More advanced automatic robotic techniques, artificial intelligence (AI), and future

DOI: 10.1016/j.ymben.2018.04.001

bio-artificial intelligence (BI) are promising for HTP assembly in the future (Ma and Huo, 2016; Nesbeth et al., 2016). These advanced methods will accelerate the assembly of genetic elements and promote the accuracy of the process in *Z. mobilis* applications.

Pathway construction and metabolic modeling for chassis design

The spatial distance between substrate and enzyme can be reduced by fixing the enzymes to a specific scaffold in metabolic pathways to improve the catalytic efficiency (Delebecque et al., 2012; Dueber et al., 2009), also referred to as metabolic channeling (Abernathy et al., 2017; Castellana et al., 2014). To date, established scaffolds include protein, DNA, and RNA. Through this modular metabolic engineering, key enzymes are organized into distinct modules and their expression is simultaneously varied to balance metabolic flux (Biggs et al., 2014; Xu et al., 2013). Modular engineering has been shown effective in enhancing the production of L-tyrosine and various terpenoids (farnesene, astaxanthin, lycopene, β-carotene, etc) in *E. coli* (Bian et al., 2017; Juminaga et al., 2012; Ma et al., 2016; Zhao et al., 2013; Zhu et al., 2014), as well as the production of N-acetylglucosamine in *Bacillus subtilis* (Liu et al., 2014b).

Cell-free metabolic engineering (CFME) can be applied to provide guidance for pathway design. For instance, precise complex biomolecular synthesis can be conducted using purified enzyme systems or crude cell lysates that can be accurately monitored and modeled (Bujara et al., 2010; Harper et al., 2012). This strategy has been successfully applied in the production of chemicals like ethanol, isobutanol, and farnesene in *E. coli* (Guterl et al., 2012; Zhu et al., 2014). In *Z. mobilis*, the kinetic modeling of the ED pathway was utilized to simulate glycolysis in cell-free extracts which has good agreement with the fluxes and steady-state intermediate concentrations (Rutkis et al., 2013). With the help of CFME, these complicated simulations will be greatly simplified in the future, and guide effecient pathway design in *Z. mobilis*. However, the incorporation of foreign genes or pathways often switches cellular metabolism or redox balance to alternative cellular metabolic and regulatory pathways that lead to the accumulation of toxic or unwanted intermediates. It is therefore essential to predict the in vivo metabolic effects of rational pathway engineering to build efficient strains for desired bioproducts.

DOI: 10.1016/j.ymben.2018.04.001

Metabolic models have shown to be particularly useful for the strain development of most industrially relevant microorganisms (Barrow et al., 1984). To date, several genome-wide metabolic models and several kinetic or stoichiometric models of central metabolism have been constructed for *Z. mobilis* to guide metabolic engineering practices (Altintas et al., 2006; Kalnenieks et al., 2014; Lee et al., 2010; Pentjuss et al., 2013; Rutkis et al., 2013; Widiastuti et al., 2011). For example, a simulation-ready model of *Z. mobilis* ED pathway comprising of only 16 enzymatic reactions was built and metabolic control analysis of this model pointed to ATP turnover as a major bottleneck due to the fact that only one ATP was produced by the ED pathway per glucose. These studies suggest that single enzymes within the ED pathway should not be considered as a prime target for overexpression to increase the glycolytic flux in *Z. mobilis* (Kalnenieks et al., 2014; Pentjuss et al., 2013; Rutkis et al., 2013). Additionally, the functional role of *pdc* and *adh* genes during ethanol production in *Z. mobilis* was confirmed via a genome-scale metabolic network (izm363) (Widiastuti et al., 2011) and a double gene knockout (*pdc* and *ldhA*) was simulated in *Z. mobilis* (ZmoMBEL601) for succinic acid overproduction through genome-scale metabolic modeling (Lee et al., 2010).

So far, three medium-scale and two genome-scale stoichiometric metabolic network models have been built for *Z. mobilis* and metabolic modeling algorithms have been continually improved to fully capture the metabolism of this organism (Kalnenieks et al., 2014; Lee et al., 2010; Pentjuss et al., 2013; Rutkis et al., 2013; Widiastuti et al., 2011). However, accurate genome-scale models reflecting the dynamic changes of cellular metabolisms for balanced cell growth and bioproduct production still need to be further developed to guide the metabolic and regulatory pathway design in *Z. mobilis*. In addition, work should be focused on shifting the main ethanol biosynthesis pathway toward production of other products in *Z. mobilis*.

Evaluation of metabolic engineering performance in *Z. mobilis*

HTP screening and characterization: Traditional approaches to measure concentrations of metabolites of interest that involve shake flasks and microtiter plates are time consuming, laborious, and costly. More importantly, they generally cannot achieve the throughput needed to efficiently screen and characterize large genetic libraries based on phenotypic or genotypic

DOI: 10.1016/j.ymben.2018.04.001

traits. Accordingly, HTP analysis is essential for detecting desired mutants from large pools of mutants that typically span ~10^5 to 10^{12} clones (Ma and Huo, 2016).

Microfluidic platforms can achieve high efficiency by using fluorescent reporters (Dietrich et al., 2010; Wang et al., 2014). Biosensors also enables HTP screen by transducing the target molecule concentration into an easily measurable signal such as fluorescence, luminescence, or absorbance (Lin et al., 2017). Likewise, techniques such as fluorescence activated cell sorting (FACS) or droplet-based microfluidic sorting such as fluorescence-activated droplet sorting (FADS) are widely utilized for HTP analysis and screening of genetic libraries. The former can be used for the detection of intracellular metabolites, or metabolites attached to the cell membrane (Dietrich et al., 2010; Yang and Withers, 2009), and the latter can be used for the detection of secretory metabolites (Kim et al., 2014; Sjostrom et al., 2014). As the reporter genes such as *gfp* and *yfp* have been utilized in *Z. mobilis* (Cho et al., 2017; Dunn and Rao, 2015; Flamholz et al., 2013), other fluorescence detection methods mentioned above can be also applied in *Z. mobilis* for HTP screen.

The Bioscreen C is an example of an HTP screening instrument capable of simultaneously monitoring two 100-well plates at 0.4 mL scale to measure cellular growth and has been widely used to obtain detailed inhibitory kinetics for *Z. mobilis* in the presence of specific compounds of interest (Franden et al., 2009; Franden et al., 2013; Yang et al., 2010a). In addition, other HTP evaluation techniques and approaches, such as Biolog's Phenotype Microarrays and BioLector systems, have also been developed and can be used in *Z. mobilis*. The Phenotype MicroArray™ protocol has already been used to profile nearly 2,000 *Z. mobilis* cellular phenotypes and has provided an overview of *Z. mobilis* physiology for optimizing the bioprocesses and detailed comparisons of other wild-type strains and mutant strains (Bochner et al., 2010).

The versatile applications of these HTP screening methods not only can be applied in *Z. mobilis* to accelerate mutant and genetic libraries screening, microbial physiology investigation, fermentation condition optimization and systems biology studies, but can also provide guidelines for subsequent test and analyze process the in metabolic engineering cycle.

Systems biology analyses: System biology (omics) analyses can offer comprehensive

DOI: 10.1016/j.ymben.2018.04.001

information to elucidate various phenomena in metabolically engineered strains. These analyses can also be helpful in identifying specific engineering targets by providing a rapid and relatively inexpensive way for performing genome-scale evaluations. Genes, sRNA, and 5' UTRs related to inhibitor tolerance have been identified and characterized in *Z. mobilis* through systems biology (Skerker et al., 2013; Yang et al., 2010a; Yang et al., 2010b). Since a previous review on systems biology studies in *Z. mobilis* was published in 2014 (He et al., 2014), quite a few new omics studies have been reported and are summarized in this review (**Table 5**). The information gathered from these systems biology studies can guide future metabolic engineering for strain improvement, facilitate genome-scale metabolic modelling, and help optimize modules in the steps of “Design” and “Build” stages for the establishment of a streamlined metabolic engineering workflow in this organism.

Concluding remarks

Z. mobilis is a natural ethanologenic bacterium with many ideal features as an industrial cell factory. Significant progress has been made to develop *Z. mobilis* for lignocellulosic biofuel and biochemical production through systems metabolic engineering approaches to expand its substrate and product ranges with improved robustness against lignocellulosic biomass hydrolysate inhibitors. In addition, biological parts of functional genes, promoters, sRNAs, and 5′ UTR have started to be identified and characterized. Reporter gene systems have also been established to characterize biological parts, as well as assembly strategies and metabolic models for rational pathway construction.

However, several challenges still need to be resolved to meet the goal of developing *Z. mobilis* as an advanced industrial chassis for economic lignocellulosic bioproducts. Some of these challenges include balanced cellular growth and productivity as well as the efficient C5/C6 co-utilization under inhibitory environments like toxic biomass hydrolysates. The industrially-relevant issues will likely be overcome by further systematic identification and characterization of functional and regulatory biological parts, continuous improvement of metabolic models that can effectively integrate omics data and advanced algorithms for pathway design in the context of dynamic cellular metabolism, and by further development of efficient and economic biological module and pathway assembly strategies, as well as HTP screening and characterization systems.

The technology advances in NGS- and MS- based omics approaches and new developments in fluxomic techniques should also prove useful in unraveling the underlying mechanisms affecting *Z. mobilis* heterologous pathway engineering. Likewise, we expect that the continual development of synthetic biology tools and genome-editing strategies will facilitate the “Design-Build-Test-Learn” cycle in *Z. mobilis* to incorporate “Bottom up” and “Top down” rational metabolic engineering strategies to ultimately meet the needs of developing *Z. mobilis* as a robust chassis for environmental, industrial, and biomedical applications (**Fig. 4**).

Abbreviations

ED pathway: Entner-Doudoroff pathway
EMP pathway: Embden-Meyerhof-Parnas pathway   ATP: adenosine triphosphate
PPP: pentose phosphate pathway   TCA cycle: tricarboxylic acid cycle
NADH: nicotinamide adenine dinucleotide (reduced)
NAD: nicotinamide adenine dinucleotide   GRAS: generally regarded as safe
NGS: next-generation sequencing   MS: mass spectrometry
ORFs: open reading frames   RNA: ribonucleic acid   DNA: deoxyribonucleic acid
mRNA: messenger RNA   CBP: consolidated bioprocessing
PHB: polyhydroxylbutyrate   2,3-BDO: 2,3-butanediol
HMF: hydroxymethylfurfural   1,3-PDO: 1,3-propanediol
iGEM: International Genetically Engineered Machine   LBA: lactobionic acid
IPTG: isopropyl β-D-1-thiogalactopyranoside   UTRs: untranslated regions
sRNAs: small RNAs   TPP: thiamine pyrophosphate   RBS: ribosome binding site
CRISPR RNAs: clustered regularly interspaced short palindromic repeat RNAs
siRNA: short interfering RNA   CRISPR-Cas system: CRISPR associated system
gTME: global transcriptional machinery engineering
GFP: green fluorescent protein   YFP: yellow fluorescent protein
HTP: High-throughput   TIGRs: tunable intergenic regions

DOI: 10.1016/j.ymben.2018.04.001

STARs: small transcription activating RNAs FACS: fluorescence activated cell sorting

FADS: droplet-based microfluidic sorting CFME: cell-free metabolic engineering

GEMs: Genome-scale metabolic models

NCD: nicotinamide cytosine dinucleotide

CMC: Carboxy Methylated Cellulose

Authors' contributions

SY and LMC conceived the outline with inputs from all authors. XW, QH, YY, and JW prepared and wrote the manuscript. SY revised the manuscript. YH, BW, MH, YZ, JB, KH and LMC conducted extensive review, and all authors contributed to the writing of the final manuscript.

Acknowledgements

LMC and KH would like to acknowledge the funding of National Science Foundation Career Award (CBET-1254754) and Welch Foundation (grant F-1756) to LMC, and Graduate Research Fellowship (DGE-1610403) to KH. YZ is funded by the DOE Great Lakes Bioenergy Research Center (DOE Office of Science BER DE-FC02-07ER64494 and DE-SC0018409).

Ethical approval

This study does not contain any studies with human participants or animals performed by any of the authors. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Legend

Table 1 Comparison of physiological features of *Z. mobilis* to *E. coli* and *S. cerevisiae*, which are mainly based on the information from previous three publications (Chen et al., 2017; Ohta et al., 1991; Panesar et al., 2006). ED: Entner-Doudoroff pathway, EMP: Embden-Meyerhof-Parnas pathway, TCA: tricarboxylic acid cycle, GRAS: generally recognized as safe, PFK: phosphofurctokinase.

Table 2 Summary of *Z. mobilis* strains with genome sequenced.

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Fig. 1. Development of *Z. mobilis* as a cell factory for lignocellulosic biofuel and biochemical production through metabolic engineering. Functional bio-parts will be selected from databases and literature. The individual bio-parts or heterologous pathway assembled from different bio-parts will be engineered into *Z. mobilis*. Optimization of the pathway or a specific bio-part will be carried out if the productivity of target bioproduct does not meet the expectation. High-throughput (HTP) screening approaches will be deployed and/or developed to select engineered strain with desired phenotype. PHB: polyhydroxybutyrate, 2,3-BDO: 2,3-butanediol.

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Categories	***Z. mobilis***	***E. coli***	***S. cerevisiae***
Growth condition	Facultative anaerobic	Facultative aerobic	Facultative aerobic
Taxonomy	Gram-negative bacterium	Gram-negative bacterium	Eukaryotic microorganism
Energy metabolism	ED pathway (1 ATP per glucose)	EMP pathway (2 ATP per glucose) and TCA	EMP pathway (2 ATP per glucose) and TCA
Ethanol productivity	5.67	0.60	0.67
Respiratory chain	Uncoupled energetics and cellular growth, high rate O_2 consumption	Coupled with cell growth, ATP accumulation inhibits PFK	Coupled with cell growth, ATP accumulation inhibits PFK
Safety status	GRAS	Not GRAS	GRAS
Theoretical yield of ethanol	98%	88% (recombinant *E. coli* (pLBA102))	90-93%
Ethanol tolerance (v/v)	16%	6%	15%
pH range	3.5-7.5	4.0-8.0	2.0-6.5
N_2 utilization	Yes	No report	No report
Median genome size	2.14 Mb	5.15 Mb	12.12 Mb

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Strains	**Genome**	**Genome Size**	**Plasmid #**	**Accession #**	**References**
ZM4 (ATCC 31821)	Complete	2.05636-Mb 2.05875-Mb 2.05875-Mb	0 5 4	NC_006526.1 (NC_006526.2) CP023715-9	Seo et al., 2005 Yang et al., 2009 Unpublished data
NCIMB 11163	Complete	2.22352-Mb	4	NC_013355.1	Kouvelis et al., 2009 So et al., 2014
ATCC 29192	Complete	2.06141-Mb	2	NC_015709.1	Kouvelis et al., 2009
ATCC 10988	Complete	2.14346-Mb	8	NC_017262.1	Pappes et al., 2011 Arvanitis et al., 2000
ATCC 29191	Complete	2.00834-Mb	3	NC_018145.1	Desiniotis et al., 2012
ZM401 (ATCC 31822)	Draft genome	2.03907-Mb	In scaffolds	AMSR00000000.1	Zhao et al., 2012
NRRL B-12526	Complete	2.17900-Mb	5	NZ_CP003709.1	Direct submission, 2012
CP4 (NRRL B-14023)	Complete	2.16324-Mb 2.14718-Mb	5 4	NC_022900.1 NZ_CP003715.1	Kouvelis et al., 2014
ZM481 (ATCC 31823)	Draft genome	2.20072-Mb	In scaffolds	LSFP00000000.1	Zhao et al., 2016
NRRL B-1960	Complete	2.04579-Mb	2	CP021053.1	Chacon-Vargas et al., 2017
8b (ZM4 derived)	Complete	2.06475-Mb	4	CP023682-6	Unpublished data
2032 (ZM4 derived)	Complete	2.07217-Mb	4	CP023677-81	Unpublished data

Table 3 List of heterologous genes used in *Z. mobilis* to expand substrate or bioproduct ranges. CBP: consolidated bioprocessing, CMC: carboxy methylated cellulose, PHB: polyhydroxybutyrate.

Gene name	**Gene Function**	**Source**	**Engineering goal**	**Yield**	**Reference**
xylA,B; tal, tktA	Xylose isomerase, Xylulokinase; Transaldolase, Transketolase	*E. coli*	Xylose utilization	0.44 g/g	Zhang and Eddy, 1995
xlyE	Xylose transporter	*E. coli*	Xylose metabolism	0.46 g/g	Dunn and Rao, 2014
araB, A, D; talB, tktA	L-ribulokinase, Arabinose isomerase, L-ribulose-5-phosphate-4-epimerase; Transaldolase, Transketolase	*E. coli*	Arabinose metabolism	0.50 g/g	Deanda et al., 1996
pmi	Phosphomannose-isomerase	*E. coli*	Mannose utilization		Weisser et al., 1996
glu	β-glucosidase	*R. albus*	CBP strain development	0.49 g/g	Yanase et al., 2005
E1,GH12	Endo-1,4-β-glucanase	*A. cellulolyticus*	CBP strain development		Linger et al., 2010
GQ368735	Endoglucanase	*E. cloacae*	CBP strain development	5.50% V/V using CMC	Vasan et al., 2011
bglB	β-glucosidase	*B. polymyxa*	CBP strain development		Luo and Bao, 2015
	Endoglucanase, Exoglucanase, and, β-glucosidase	*T. reesei*	CBP strain development	9.50% V/V using CMC	Venkatesh, 2015
alaD	L-alanine dehydrogenase	*B. sphaericus*	Alanine production	7.50 g/L	Uhlenbusch et al., 1991
crtB,crtE, crtI, crtY	Carotenoid biosynthetic genes	*E. uredovora*	β-carotene production	220 μg/g	Misawa et al., 1991
phbCAB	Polyhydroxyalkanoate synthase, β-ketothiolase, Acetoacetyl- CoA reductase	*R. eutropha*	PHB accumulation		Lai and Chen, 2006
kivd, adhA	2-ketoisovalerate decarboxylase, Alcohol dehydrogenase	*L. lactis*	Isobutanol production		He et al., 2014

als, aldC, bdh	Acetolactate synthase, Decarboxylase, Butanediol dehydrogenase	*B. licheniformis/E. cloacae*	2,3-butane diol production	>10 g/L	Yang et al., 2016b
dldh	D-lactate dehydrogenase	*Leuconostoc sp.*	D-lactate production	65.69 g/L	Kim et al., 2016

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Table 4 List of genes responsible for inhibitor tolerance improvement in *Z. mobilis*.

Gene	**Function**	**Host**	**Technique(s)**	**Resistance**	**Reference**
himA (*ZMO1122*)	Aldo/keto reductase	ZM4	Reduced expression	Potassium acetate (8.00 g/L)	Viitanen et al., 2009;
hfq (*ZMO0347*)	Global regulator, RNA-binding protein	ZM4	Overexpression	Acetate, vanillin, furfural, and HMF	Yang et al., 2010b
nhaA (*ZMO0119*）	Sodium proton antiporter	ZM4	Overexpression	Sodium acetate	Yang et al., 2010a; Liu et al., 2017
ZMO0128	TonB-dependent receptor	ZM4	Disruption	Ammonium acetate (15.00 g/L)	Yang et al., 2014a
ZMO1116, ZMO1696, ZMO1885	Glutamate synthase, Alcohol dehydrogenase, and NADH oxidase	ZM4	Overexpression	4-hydroxybenzaldehyde, vanillin	Yi et al., 2015
ZMO1598, ZMO1234	1-deoxy-D-xylulose-5-phosphate synthase，	8b	Disruption	Furfural (<3.00 g/L)	Yang et al., 2014b
ZMO0282, ZMO0283, ZMO0285	Efflux transporter, Hydrophobe/amphiphile Efflux-1 (HAE1) family, Outer membrane lipoprotein	8b	Disruption	Furfural	Yang et al., 2014b
ZMO0281	Transcriptional regulator, TetR family	8b	Overexpression	Furfural	Yang et al., 2014b
ropD	Sigma factor 70	ZM4	Modified *ropD*	Furfural (<3.00 g/L)	Tan et al., 2015
irrE	Regulatory protein	*E. coli*	Heterologous engineering	NaCl (175.50 g/L)	Zhang et al., 2010
ndh (*ZZ6_0213*)	Type II NADH dehydrogenase	ZM6	Overexpression	NaCl (<14.63 g/L)	Hayashi et al., 2015

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cydAB (*ZZ6_1531*, *ZZ6_1532*)	Terminal cytochrome bd-type ubiquinol oxidase	ZM6	Overexpression	NaCl (<13.16 g/L)	Hayashi et al., 2015
himA (*ZMO1122*)	Aldo/keto reductase	ZM4	Disruption	NaCl (20.00 g/L)	Wang et al., 2016
ZMO1875	Unknown protein DUF1476	ZM4	Overexpression	Miscanthus hydrolysate	Skerker et al., 2013
ZMO1162	Histidine kinase	33C and 8b	Overexpression	Hydrolysate (100-1,000 folds more)	Yang et al., 2014b
ZMO0038	Sigma 54 modulation protein	33C and 8b	Disruption	Hydrolysate (100-1,000 folds more)	Yang et al., 2014b
fdh	Formate dehydrogenase gene	*S. cerevisiae*	Heterologous engineering	Corn stover hydrolysate	Dong et al., 2013
ZMO1771	Alcohol dehydrogenase	ZM4	Overexpression	Corn stover hydrolysate	Wang et al., 2017

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Table 5 Summary of systems biology studies carried out in *Z. mobilis*. RM: rich medium, MM: Minimum medium, Temp.: temperature, LBNL: Lawrence Berkeley National Laboratory, NREL: National Renewable Energy Laboratory, CGS: comparative genome resequencing, NGS: next-generation sequencing. NA: data not available.

Strains	**Growth Condition**				**Omics-type(s)**	**Accession #**	**Reference**
	Temp.	**Media**	**Inhibitor or Exp. Goal**	**Growth Phase**			
ZM4	30 °C	RM (2% glucose)	Anaerobic or Aerobic	3, 26 h	Expression array	GSE10302	Yang et al., 2009b
ZM4	30 °C	RM (2% glucose)	Aerobic or Anaerobic	13, 26 h	sRNA NGS	GSE57773	Cho et al., 2014
ZM4	30 °C	RM (2.5% glucose) MM (2% glucose)	Heat shock 1-h at 30°C	Mid-exponential	Tiling array	GSE51870	Deutschbauer et al., 2014
ZM4	30 °C	RM with or without 10 mM sorbitol	Glucose (220 g/L)	1) 2% glucose 6 h 2) 22% glucose at (6+2) h and (6+14) h 3) 22% glucose with sorbitol at (6+12) h	Expression array	GSE49620	Zhang et al., 2015
ZM4	30 °C	RM (2% glucose)	Ethanol (40 g/L)	24 h	Expression array	GSE39558	He et al., 2012
ZM4	30 °C	RM	Ethanol (47 g/L)	6, 10, 13.5, and 26 h	Expression array	GSE21165	Yang et al., 2013

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ER79ap ER79ag (ZM4 mutants)	30 °C	MR-MES (Bringer et al., 1985)	Ethanol (70g/L)	Mid-log phase	Expression array	GSE90043	Universidad Nacional Autonoma de Mexico, Unpublished
ZM4	30 °C	RM (2% glucose)	Furfural (1 g/L)	24 h	Expression array	GSE37848	He et al., 2012
8b	33 °C	RM (8% glucose or 8% xylose)	Furfural shock (3 g/L), Furfural stress (2 g/L)	Exponential	Tiling array	GSE63540	NREL, Unpublished
AcR (ZM4 mutant)	30 °C	RM (2% glucose)	Sodium acetate (12 g/L)	Exponential	CGS	GSE18106	Yang et al., 2010
AcR (ZM4 mutant)	30 °C	MM	Sodium acetate (10 g/L)	130, 148, 166, and 190 h	Expression array Proteomics	GSE25443	Yang et al., 2014a
8b	33 °C	RM (8% glucose, 8% xylose, or 4% glucose and 4% xylose)	Acetate	Exponential, Transition, and Stationary	Tiling array	GSE57553	Yang et al., 2014b
#7 (8b mutant)	33 °C	RM (5% xylose)	Xylose utilization	Exponential	CGS	NA	Mohagheghi et al., 2014
ZM4	30 °C	RM (2% glucose)	5 mM 4-hydroxybenzal	Every 4-h till 36 h	Expression array	NA	Yi et al.,

			dehyde, Syringaldehyde, or Vanillin				2015
ZM4, ZM401	NA	RM (2.5% glucose) MM (2% glucose)	Various inhibitors	Stationary	Expression array Metabolomics	GSE 39466	Deutschbauer et al., 2014
SS3 (8b mutant)	33 °C	Pretreated corn stover liquor	Corn stover hydrolysate	Exponential	CGS	NA	Mohagheghi et al., 2015
KLD 1, 2 (ZM4 mutants)	30 °C	RM (10% xylose) or RM (2.5% arabinose)	Pentose utilization of xylose and arabinose	NA	CGS	NA	Dunn et al., 2015
ZM4, RDM-4 (ATCC29191 mutant)	30 °C, 38 °C	NA	Aerobic fermentation and thermotolerance	Late-exponential	Expression array	GSE 22355	Beppu university, Unpublished
ZM401	30 °C	RM (5% glucose)	Flocculent behavior	Exponential	CGS, Expression array	NA	Jeon et al., 2012
ZAG-12	30 °C	100.0 g/L sucrose	Global profile of regulatory proteins	24 h	Proteomics	NA	Cavalcanti et al., 2015

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Panesar, P.S., Marwaha, S.S., Kennedy, J.F., 2006. *Zymomonas mobilis*: an alternative ethanol producer. J. Chem. Technol. Biotechnol. 81, 623-635.

Highlights:

- *Z. mobilis* has attractive physiological features for lignocellulosic biofuels and biochemicals.
- Significant progress achieved in *Z. mobilis* for expanded substrate utilization and biochemical production as well as robustness improvement.
- Knowledge learnt from *Z. mobilis* can be employed in metabolic engineering of other microorganisms.
- *Z. mobilis* can be developed as a synthetic chassis cell with progress accomplished such as the systemical identification and characterization of biological parts.

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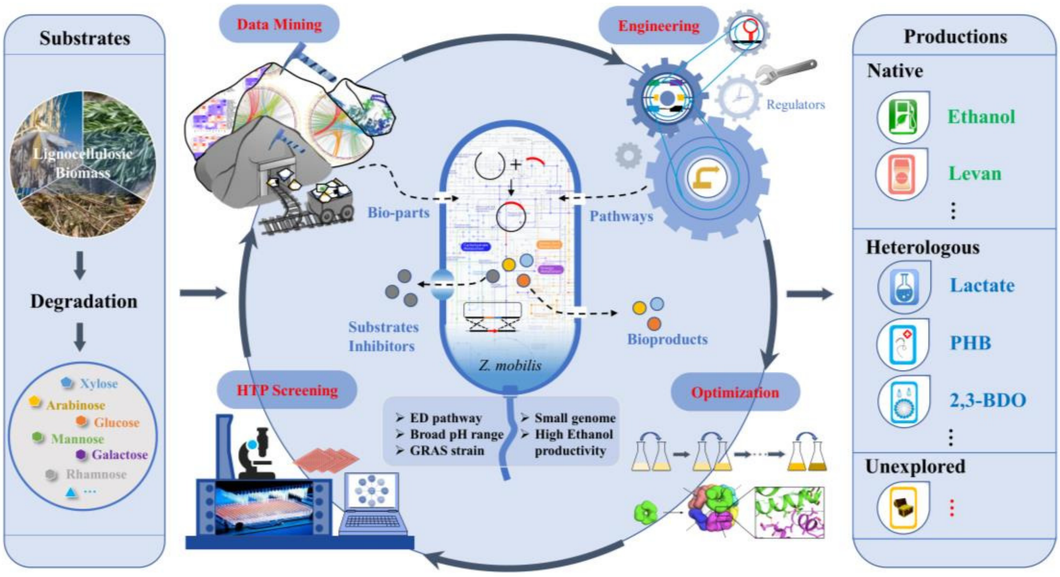

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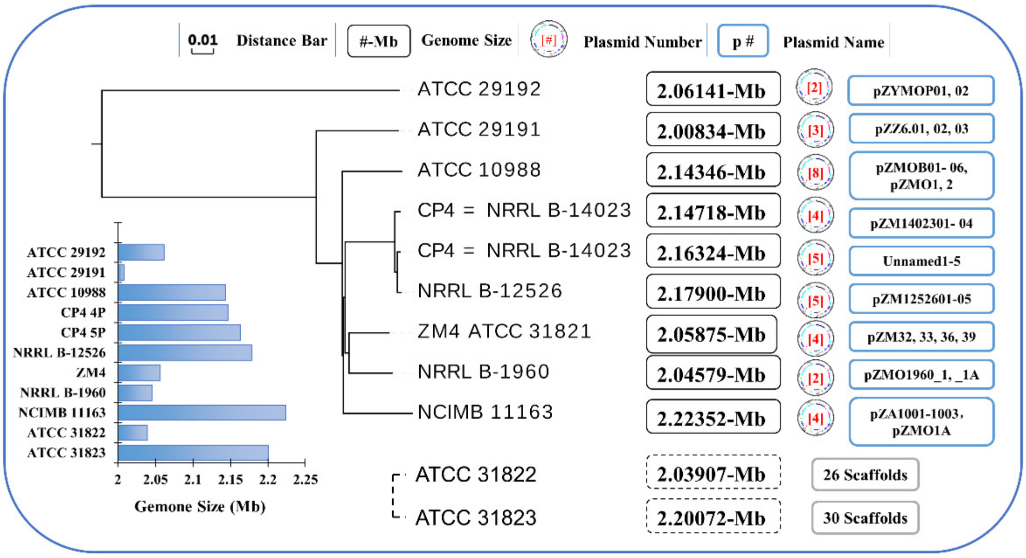

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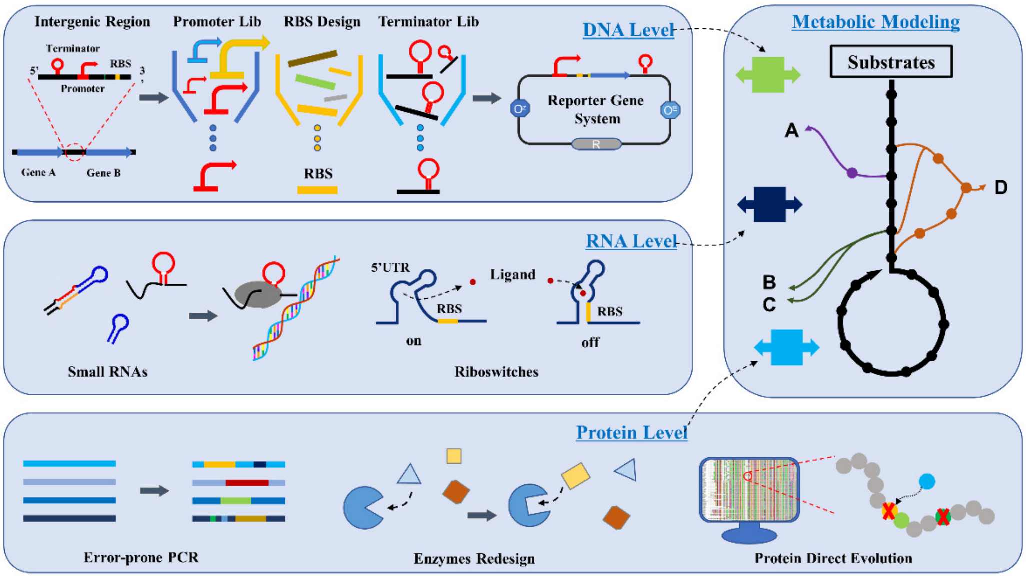

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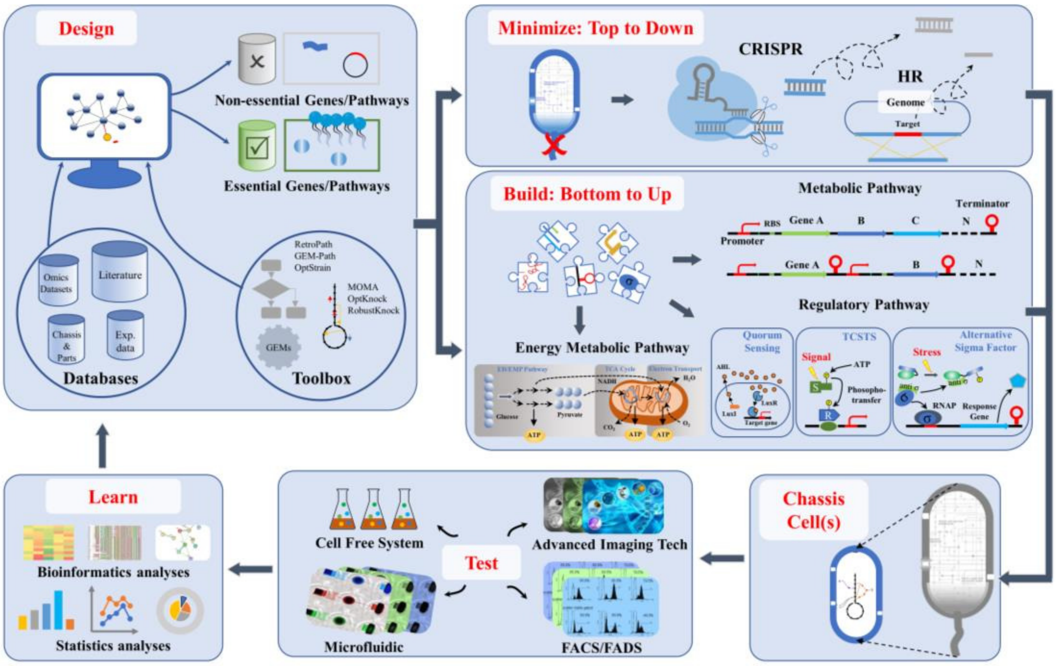

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