Advances and prospects in metabolic engineering of *Zymomonas mobilis*

Xia Wang, Qiaoning He, Yongfu Yang, Jingwen Wang, Katie Haning, Yun Hu, Bo Wu, Mingxiong He, Yaoping Zhang, Jie Bao, Lydia M. Contreras, Shihui Yang

**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.

**1. Introduction**

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 biorefineries applications.

**2. Attractive physiological features of Z. mobilis for biotechnology**

*Z. mobilis* is a natural facultative anaerobic ethanologenic Gram-negative bacterium with many desirable industrial characteristics...
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fl 
al metabolism. Notably, the EMP pathway, pentose phosphate
pathway, and TCA cycle are less thermodynamically con- 

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: phosphofructokinase.

<table>
<thead>
<tr>
<th>Categories</th>
<th>Z. mobilis</th>
<th>E. coli</th>
<th>S. cerevisiae</th>
</tr>
</thead>
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<tr>
<td>Growth condition</td>
<td>Facile anaerobic</td>
<td>Facile aerobic</td>
<td>Facile aerobic</td>
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<tr>
<td>Taxonomy</td>
<td>Gram-negative bacterium</td>
<td>Gram-negative bacterium</td>
<td>Eukaryotic microorganism</td>
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<td>Energy metabolism</td>
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<td>EMP pathway (2 ATP per glucose) and TCA</td>
<td>EMP pathway (2 ATP per glucose) and TCA</td>
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<td>0.60</td>
<td>0.67</td>
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<td>Respiratory chain</td>
<td>Uncoupled energetics and cellular growth, high rate O2 consumption</td>
<td>Coupled with cell growth, ATP accumulation inhibits PFK</td>
<td>Coupled with cell growth, ATP accumulation inhibits PFK</td>
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<tr>
<td>Safety status</td>
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<td>Not GRAS</td>
<td>GRAS</td>
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<td>90-93%</td>
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<td>Median genome size</td>
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<td>5.15 Mb</td>
<td>12.12 Mb</td>
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(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 mol of ATP is yielded per mol 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, phosphofructokinase (Pfk) in the EMP pathway, phosphoglucone dehydrogenase (Pgd) and transaldolase (Tal) in the PPP pathway, as well as 2-oxoglutarate dehydrogenase complex (su-
fl 
cABCD) 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; Kalenieks et al., 2008; Rutkis et al., 2014, 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, 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.
**Z. mobilis** an ideal host for industrial biotechnology applications.

3. 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** strains. Since the initial genome sequence of model species ZM4 was published in 2005 and its annotation improved in 2009 (See et al., 2005; Yang et al., 2009a), several other strains have been sequenced, including CP4, NCIBM 11163, ATCC 29191, ATCC 29192, ATCC 10988, ATCC 31822, ATCC 10988, ATCC 31823, NRRL B-12526, and NRRL B-1256 (Chacon-Vargas et al., 2017; Desniotis et al., 2012; Kouvelis et al., 2011; Zhao et al., 2012, 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 (See 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 (single nucleotide polymorphisms) and a 2400-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 (Widiatutti et al., 2011).

With the availability of various **Z. mobilis** genomes, systems biology studies have provided guidelines for strain improvement (He et al., 2012a, 2012b; Skerker et al., 2013; Yang et al., 2014a, 2010a, 2014b, 2013, 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; Widiatutti et al., 2011).

4. 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, 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), 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 Gif in \textit{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 \textit{E. coli} in \textit{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, \textit{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). As a way to address this, expression of a low-affinity xylose transporter XlyE from \textit{E. coli} in \textit{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, \textit{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 \textit{Z. mobilis} have been identified, ethanol production from lignocellulosic pentose sugars by the engineered \textit{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 \textit{Z. mobilis} must be improved further especially when lignocellulosic biomass hydrolysates containing toxic compounds such as furfural and acetate are used.

5. Construction of \textit{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, \textit{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 \(\beta\)-glucosidase (EC3.2.1.21). Except that the enzymatic activity of \(\beta\)-1,4-endoglucanase (encoded by ZMO1086) was determined by a previous study (Rajnish et al., 2008), \textit{Z. mobilis} lacks of most cellulolytic enzymes.

Different cellulolytic enzymes from other species have thus been expressed in \textit{Z. mobilis}, and the resulting recombinant strains have been demonstrated to be able to ferment cellulosic polysaccharides directly into ethanol (Table 3). For example, \textit{Z. mobilis} has been engineered to ferment cellulose with a yield of 0.49 g ethanol/g substrate by expressing a heterologous \(\beta\)-glucosidase from \textit{Ruminococcus albus} with a native signal peptide (Yanase et al., 2005). Heterologous endo-1,4-\(\beta\)-\(D\)-glucansases, such as two enzymes of \textit{E. coli} and \textit{GH12} from \textit{Acidothermus cellulolyticus} or endo-1,4-\(\beta\)-D-glucanase from \textit{Entero bacter cloacae}, have also been successfully expressed in \textit{Z. mobilis} (Linger et al., 2010; Thirumalai Vasan et al., 2011). Subsequently, a heterologous \(\beta\)-D-glucosidase from \textit{Bacillus polymyxa} has been expressed in \textit{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 \(\beta\)-glucosidase from \textit{Trichoderma reesei} have been transformed into \textit{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 \textit{Z. mobilis} can be a promising CBP platform microorganism with the capacity to express and secrete cellulolytic enzymes through metabolic engineering, other issues need to be resolved to make \textit{Z. mobilis} 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 \textit{Z. mobilis} CBP strains for commercial biofuel production considering that only 1 mol of ATP is produced per mole of glucose. Therefore, metabolic engineering efforts in \textit{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).

6. Engineering \textit{Z. mobilis} for a variety of biochemical products

Other than ethanol, \textit{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 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 \textit{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, \textit{alaD} gene encoding \(\gamma\)-alanine dehydrogenase from \textit{Bacillus sphaericus} has been cloned and introduced into \textit{Z. mobilis}, leading to the production and excretion of 7.5 g/L \(\gamma\)-alanine by this recombinant strain (Uhlenbusch et al., 1991). Likewise, a recombinant \textit{Z. mobilis} strain with imported D-lactate dehydrogenase genes from \textit{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 \textit{phbCAB} from \textit{Ralstonia eutropha} has been engineered into \textit{Z. mobilis} and enzymatic activities of PHB \(\beta\)-ketohydrolase and PHB (acetocetyl-CoA reductase) have been detected with PHB accumulation (Lai and Chen, 2006). In a different study, the expression of four carotenoid biosynthetic genes (\textit{crbT}, \textit{crf1}, \textit{crf2}, \textit{crf3}) in \textit{Z. mobilis} from \textit{Erwinia uredovora} resulted in the production of \(\beta\)-carotene (Misawa et al., 1991).

In addition, an engineered \textit{Z. mobilis} ZM4 strain has also been

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**Fig. 2.** Genomic differences and relationships among different \textit{Z. mobilis} strains that have been sequenced. The evolutionary tree (solid line section) was built using the CVTree3.0 web-server with all protein sequences of each \textit{Z. mobilis} strain that have been sequenced. The genome sequences of ATCC 31822 and 31823 are not completely finished containing 26 and 30 scaffolds respectively, which are connected by a dotted line in the middle of the graph bottom. The histogram on the bottom left represents the genome size of each sequenced \textit{Z. mobilis} strain.
7. 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 by-products 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.

8. Engineering Z. mobilis for improved robustness

Besides the toxic end-products such as ethanol produced during fermentation, other 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 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 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 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.
Table 4

<table>
<thead>
<tr>
<th>Gene</th>
<th>Host</th>
<th>Function</th>
<th>Resistance</th>
<th>Technique(s)</th>
<th>Ref.</th>
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<td>fdh</td>
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<td>Formate dehydrogenase gene</td>
<td>Overexpression</td>
<td>ZM4</td>
<td>Joachimsthal et al. (1998)</td>
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<tr>
<td>cydAB</td>
<td>ZZ6_1531 ZZ6_1532</td>
<td>Terminal cytochrome bd-type ubiquinol oxidase</td>
<td>Overexpression</td>
<td>ZM6</td>
<td>Hayashi et al. (2015)</td>
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Previous studies have observed that, during growth, Z. mobilis is capable of converting 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 these 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 (Franden et al., 2013; Gu et al., 2015; Yi et al., 2015). 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, 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 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 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).

### 9. 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 sucrose SacC (ZMO0375, EC 3.2.1.26), and glucose-facilitated diffusion protein Gif (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 terminal of Pdc to N terminal of AdhB, also 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 adh 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-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, 2010b).

### 10. 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”.

#### 10.1. Selection and characterization of biological parts for metabolic engineering in Z. 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 bio-product to a specified level under defined growth conditions in the chassis cell (Fig. 3).

#### 10.1.1. 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 an 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 been investigated in Z. mobilis. For example, P_hsc, P_hsa, P_t7, P_hsd, and P_es 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 sequenced to establish common molecular features that may specify their efficient transcription (Barnell et al., 1990; Conway et al., 1991, 1987b, 1987c; Conway and Ingram, 1988). 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., 1992).
sRNA regulatory function depends on base pairings between the sRNA subset of noncoding RNAs that can be both activators and repressors for gene expression in bacteria. sRNAs in prokaryotes are relatively short regulatory small RNAs (sRNAs) have emerged as key regulators of gene expression (Mellin and Cossart, 2015; Zhang et al., 2015a). In a similar way, sRNAs have been used to construct biosensors for biotechnology applications such as sRNAs and riboswitches which will be identified and characterized for efficient metabolic flux control. At the protein level, functional bio-parts will be optimized using directed evolution strategies to change substrate binding pocket(s) or reorganize enzyme active sites. These bio-parts can be combined to construct tunable metabolic pathways for balanced cell growth and optimized bioproduct production. miRNAs: ribosome binding site. 5′ UTR: 5′ untranslated region.

10.1.2. 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., in press). 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 Storoz, 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 expression (Chen and Gottesman, 2014), and have been used to construct biosensors for biotechnology applications (Mellin and Gossart, 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 (50–300 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 (Repoli 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 (Gossinger and Hartmann, 2012; Necheosthan 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., in press).

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., 2011); 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, 2014). Among 36 regulatory 5′ UTRs candidates studied under ethanol (5% or 6%, v/v), acetate (10 g/L), and xylose (10 g/L), a few RNAs (P_{5}\text{gap}, P_{rno}, P_{pdx}, and P_{uag}) for the expression of heterologous genes in Z. mobilis (Mackenzie et al., 1989; Reynen et al., 1990; Zhang 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 has allowed the use of strong and constitutive promoters within this pathway (especially P_{gap}, P_{rno}, P_{pdx}, and P_{uag}) for the expression of heterologous genes in Z. mobilis (Mackenzie et al., 1989; Reynen et al., 1990). Among the 36 regulatory 5′ UTRs, 15 novel sRNAs were identified and its target mRNA. The base paring of sRNA-mRNA primarily occurs in the 5′ UTRs of mRNAs and most often involves in RBS (Repoli 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 (Gossinger and Hartmann, 2012; Necheosthan 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., in press).

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Fig. 3. Strategies at DNA, RNA, and protein levels to identify biological parts that can be applied in metabolic engineering practices in Z. mobilis. At the DNA level, the characterization and selection of promoter, RBS, and terminator libraries can provide DNA bio-parts with varying strength and regulation, which can be determined by reporter gene systems. At the RNA level, regulatory RNA libraries such as sRNAs and riboswitches will be identified and characterized for efficient metabolic flux control. At the protein level, functional bio-parts will be optimized using directed evolution strategies to change substrate binding pocket(s) or reorganize enzyme active sites. These bio-parts can be combined to construct tunable metabolic pathways for balanced cell growth and optimized bioproduct production. RBS: ribosome binding site. 5′ UTR: 5′ untranslated region.
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 and engineering.

10.1.3. 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., 2000; 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., 2010a, 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 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 Mg²⁺) 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 subject to site-directed mutagenesis and the enzyme activity and transfructosylation products were affected (Li et al., 2011, 2008). Moreover, random mutagenesis was performed on AdhB and variant forms were generated with increased thermostability and altered substrate specificity (Rellos et al., 1997, 1998, 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.
investigations of DNA restriction-modification (R-M) systems in \textit{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 be assembled, these new methods have improved efficiency, fidelity, and modularity, leading to simple design and benchside 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 been successfully applied in \textit{Z. mobilis} to develop an \textit{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 \textit{Z. mobilis}. This shuttle vector contains only the essential elements: replication origins for both \textit{E. coli} and \textit{Z. mobilis}, an antibiotic marker of the spectinomycin resistance gene \textit{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 \textit{Z. mobilis} were cloned for 36 candidates right in front of the \textit{gfp} gene in frame for the construction of \textit{GFP}-reporter plasmids with 5’ UTR (Cho et al., 2017). Initial implementations of these assembly methods have been useful in \textit{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 JS 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 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 \textit{Z. mobilis} applications.

10.3. 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 \textit{L}-tyrosine and various terpenoids (farnesene, astaxanthin, lycopene, \textit{\beta}-carotene, etc.) in \textit{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 2-acetylglucosamine in \textit{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 \textit{E. coli} (Guterl et al., 2012; Zhu et al., 2014). In \textit{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 efficient pathway design in \textit{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.

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 \textit{Z. mobilis} to guide metabolic engineering practices (Altintas et al., 2006; Kalnienieks 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 \textit{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 enzyme within the ED pathway should not be considered as a prime target for overexpression to increase the glycolytic flux in \textit{Z. mobilis} (Kalnienieks et al., 2014; Pentjuss et al., 2013; Rutkis et al., 2013). Additionally, the functional role of \textit{pad} and \textit{adh} genes during ethanol production in \textit{Z. mobilis} was confirmed via a genome-scale metabolic network (izm363) (Widiastuti et al., 2011) and a double gene knockout (\textit{pad} and \textit{ldhA}) was simulated in \textit{Z. mobilis} (ZmoMBE601) 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 \textit{Z. mobilis} and metabolic modeling algorithms have been continually improved to fully capture the metabolism of this organism (Kalnienieks 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 \textit{Z. mobilis}. In addition, work should be focused on shifting the main ethanol biosynthesis pathway toward production of other products in \textit{Z. mobilis}.

10.4. Evaluation of metabolic engineering performance in \textit{Z. mobilis}

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

Microfluidic platforms can achieve high efficiency by using fluorospectors (Dietrich et al., 2010; Wang et al., 2014). Biosensors
### Table 5

<table>
<thead>
<tr>
<th>Strains</th>
<th>Growth condition</th>
<th>Inhibitor or Exp. goal</th>
<th>Growth phase</th>
<th>Omics-type(s)</th>
<th>Accession #</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>ZM4</td>
<td>30 °C</td>
<td>RM (2% glucose)</td>
<td>Anaerobic or Aerobic</td>
<td>3, 26 h</td>
<td>Expression array</td>
<td>GSE10302, Yang et al. (2009b)</td>
</tr>
<tr>
<td>ZM4</td>
<td>30 °C</td>
<td>RM (2% glucose)</td>
<td>Aerobic or Anaerobic</td>
<td>13, 26 h</td>
<td>sRNA NGS</td>
<td>GSE57773, Cho et al. (2014)</td>
</tr>
<tr>
<td>ZM4</td>
<td>30 °C</td>
<td>MM (2% glucose)</td>
<td>Heat shock 1-h at 30 °C</td>
<td>Mid-exponential</td>
<td>Tiling array</td>
<td>GSE51870, Deutscher et al. (2014)</td>
</tr>
<tr>
<td>ZM4</td>
<td>30 °C</td>
<td>RM with or without 10 mM sorbitol</td>
<td>Glucose (220 g/L)</td>
<td>Expression array</td>
<td>GSE49620, Zhang et al. (2015a, 2015b)</td>
<td></td>
</tr>
<tr>
<td>ZM4</td>
<td>30 °C</td>
<td>RM (2% glucose)</td>
<td>Ethanol (40 g/L)</td>
<td>24 h</td>
<td>Expression array</td>
<td>GSE39558, He et al. (2012)</td>
</tr>
<tr>
<td>ZM4</td>
<td>30 °C</td>
<td>RM (2% glucose)</td>
<td>Ethanol (47 g/L)</td>
<td>6, 10, 13.5, and 26 h</td>
<td>Expression array</td>
<td>GSE21165, Yang et al. (2013)</td>
</tr>
<tr>
<td>ZM4</td>
<td>30 °C</td>
<td>MR-MES</td>
<td>Ethanol (70 g/L)</td>
<td>Mid-log phase</td>
<td>Expression array</td>
<td>GSE90043, Unpublished data</td>
</tr>
<tr>
<td>ZM4</td>
<td>30 °C</td>
<td>RM (2% glucose)</td>
<td>Furfural (1 g/L)</td>
<td>24 h</td>
<td>Expression array</td>
<td>GSE37848, He et al. (2012a)</td>
</tr>
<tr>
<td>ZM4</td>
<td>30 °C</td>
<td>MM</td>
<td>Sodium acetate (12 g/L)</td>
<td>Exponential</td>
<td>Tiling array</td>
<td>GSE63540, Unpublished data</td>
</tr>
<tr>
<td>ZM4</td>
<td>30 °C</td>
<td>MM</td>
<td>Sodium acetate (10 g/L)</td>
<td>130, 148, 166, and 190 h</td>
<td>Expression array</td>
<td>GSE25443, Yang et al. (2014a)</td>
</tr>
<tr>
<td>8b</td>
<td>33 °C</td>
<td>RM (8% glucose, 8% xylose, or 4% glucose and 4% xylose)</td>
<td>Acetate</td>
<td>Exponential, Transition, and Stationary</td>
<td>CGS</td>
<td>GSE377553, Mohagheghi et al. (2014)</td>
</tr>
<tr>
<td>ZM4, ZM401</td>
<td>30 °C</td>
<td>RM (5% xylose)</td>
<td>Xylose utilization</td>
<td>Exponential</td>
<td>NA</td>
<td>Yi et al. (2015)</td>
</tr>
<tr>
<td>ZM4, ZM401</td>
<td>30 °C</td>
<td>RM (2% glucose)</td>
<td>5 mM 4-hydroxybenzaldehyde, Syringaldehyde, or Vanillin</td>
<td>Exponential</td>
<td>NA</td>
<td>Expression array, Metabolomics</td>
</tr>
<tr>
<td>SSi (8b mutant)</td>
<td>33 °C</td>
<td>Pretreated corn stover liquor</td>
<td>Gorn stover hydrolysate</td>
<td>Stationary</td>
<td>NA</td>
<td>Mohagheghi et al. (2015)</td>
</tr>
<tr>
<td>ZM4, RDM-4 (ATCC29191 mutant)</td>
<td>30 °C, 38 °C</td>
<td>NA</td>
<td>Aerobic fermentation and thermotolerance</td>
<td>Late-exponential</td>
<td>Expression array</td>
<td>GSE22355, Dunn and Rao (2015)</td>
</tr>
<tr>
<td>ZM401</td>
<td>30 °C</td>
<td>RM (5% glucose)</td>
<td>Flocculent behavior</td>
<td>Exponential</td>
<td>NA</td>
<td>Jeon et al. (2012)</td>
</tr>
<tr>
<td>ZAG-12</td>
<td>30 °C</td>
<td>100.0 g/L sucrose</td>
<td>Global profile of regulatory proteins</td>
<td>24 h</td>
<td>Proteomics</td>
<td>NA</td>
</tr>
</tbody>
</table>
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, 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 2000 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 in the metabolic engineering cycle.

10.4.2. Systems biology analyses

System biology (omics) analyses can offer comprehensive 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 (Cho et al., 2014, 2017; Skerker et al., 2013; Yang et al., 2010a, 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 modeling, and help optimize modules in the steps of “Design” and “Build” stages for the establishment of a streamlined metabolic engineering workflow in this organism.

11. 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).

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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, YJ, KB, and LMC conducted extensive review, and all authors contributed to the writing of the final manuscript.

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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