Development and Characterization of Acidic-pH Tolerant Mutants of Zymomonas mobilis through Adaptation and Next Generation Sequencing based Genome Resequencing and RNA-Seq

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Research

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Abstract

Background: Acid pretreatment is a common strategy used to break down lignocellulosic biomass as substrate for biochemical production, which however generates inhibitory compounds and results in acidic pH condition. Although the natural ethanologenic bacterium Zymomonas mobilis can grow in a broad pH range, cell growth and ethanol fermentation are still affected at acidic pH conditions below pH 4.0.

Results: In this study, adaptive laboratory evolution (ALE) strategy was applied to adapt Z. mobilis under acidic pH condition. Two mutant strains named 3.6M and 3.5M with enhanced acidic-pH tolerance were selected and confirmed. Mutant strains 3.6M and 3.5M exhibited 50~130% enhancement on growth rate, 4~9 h reduction on fermentation time, and 20~63% improvement on ethanol productivity than wild-type ZM4 at pH 3.8. Next-generation sequencing (NGS)-based whole genome resequencing (WGR) and RNA-Seq technologies were applied to unravel the acidic pH tolerance mechanism of mutant strains. WGR result indicated that mutations in four genes ZMO0421 (Ala67Thr), ZMO0712 (Gly539Asp), ZMO1432 (Pro480Leu), and ZMO1733 (Thr7Lys) with non-synonymous amino acid changes might be related with the acidic-pH tolerance. Additionally, RNA-Seq result showed that the upregulation of genes involved in glycolysis and the downregulation of mobility related genes would help generate and redistribute cellular energy to help resist acidic pH while keep normal biological processes in Z. mobilis . Moreover, genes involved in RND efflux pump, ATP-binding cassette (ABC) transporter, proton consumption, and alkaline metabolite production were significantly upregulated in mutants under acidic condition compared with ZM4, which could help maintain the pH homeostasis in mutant strains for low acidic-pH resistance. Furthermore, our results also demonstrated that genes related to branch amino acid biosynthesis from threonine to isoleucine were significantly upregulated in mutant 3.6M under acidic condition compared with ZM4, and genes encoding F 1 F 0 ATPase to pump excess protons out of cells were upregulated in mutant 3.6M under pH 3.8 compared with pH 6.2. These differences could help mutant 3.6M manage acidic condition better than ZM4. A few gene targets were then selected for genetics study to confirm their role on acidic pH tolerance, and our results demonstrated that the expression of two operons in the shuttle plasmids could help Z. mobilis tolerate acidic pH condition, which are ZMO0956-ZMO0958 encoding cytochrome bc1 complex and ZMO1428-ZMO1432 encoding RND efflux pump.

Conclusion: Two acidic-pH tolerant mutants 3.6M and 3.5M obtained through this study especially 3.6M can be used as candidate strains for commercial bioethanol production under acidic fermentation conditions, and molecular mechanism of acidic pH tolerance of Z. mobilis \was further proposed, which can facilitate future research on rational design of synthetic microorganisms with enhanced tolerance against acidic pH conditions. In addition, the strategy developed in this study combining approaches of ALE, genome resequencing, RNA-Seq and classical genetics study for mutant evolution and characterization can be applied in other industrial microorganisms.

Background

With the growing demand for energy and global climate change caused by burning fossil fuels [1, 2], sustainable bioenergy has drawn the great attention [3]. Currently, bioethanol, an environmental-friendly renewable liquid biofuel, has been intensively studied as one of the most promising alternatives to fossil fuels [4, 5]. Bioethanol is primarily produced from food crops with high content of sugar and starch, which would compete with the food supply and potentially lead to global food crisis.

Lignocellulosic materials, mainly from agriculture wastes or forestry residues, are the most abundant, low-cost and promising feedstocks for bioethanol production [6, 7]. However, these biomass resources are naturally recalcitrant, which require deconstruction processes to breakdown the rigid structure to release fermentable sugars for subsequent microbial fermentation [8, 9]. Among different pretreatment methods, acid hydrolysis is an effective strategy to deconstruct lignocellulose but could lead to the generation of inhibitory compounds and acidic-pH environment [10], which consequently impedes cell growth and decreases the ethanol productivity of fermenting microorganisms [11, 12].

To minimize the detrimental effect of acidic pH on microbes, hydrolysates must be neutralized by high-cost processes such as extra chemical addition before enzyme hydrolysis and fermentation, especially in large industrial scales [13]. Whereas, a natural acidic pH condition of hydrolysate provides an opportunity to effectively prevent the potential bacterial contamination and makes the open (non-sterilized) fermentation applicable [14, 15]. It is reported that ethanol production under non-sterilized condition can save 30 ~ 40% energy consumption and make process simpler [16]. Hence, it will be ideal to develop acidic pH tolerant strains for ethanol production, which has been developed in many species, such as Escherichia coli [17, 18] and yeast [13, 19, 20].

Zymomonas mobilis is a natural ethanologenic bacterium with desirable industrial biocatalyst characteristics, such as highly specific rate of sugar uptake, high ethanol yield, non-requirement of controlled addition of oxygen and relatively low biomass production during fermentation [21, 22]. In addition, Z. mobilis has a generally regarded as safe (GRAS) status [23, 24]. However, acidic pH condition is still a challenge for Z. mobilis using lignocellulosic feedstock hydrolyzed by acid as the substrate. Up to now, many different stress-tolerant strains of Z. mobilis have been constructed with enhanced tolerance to acetate [25, 26], furfural [27, 28], and hydrolysate [29, 30]. Among these studies, only a few were focused on acid tolerance. Z. mobilis NS-7 is an acid-tolerant strain developed by nitrosoguanidine (NTG) mutation and acid medium selection, which can ferment at an acidic pH of 4.5 under non-sterilized condition without being contaminated [16]. Z. mobilis GZNS1 is another mutant strain evolved by culturing at pH 4.0 condition that could produce ethanol from acidic kitchen garbage [15]. An increased acid tolerance was also observed in Z. mobilis recombinant strain carrying Pbp (proton-buffering peptide, Pbp) from E. coli [31].

In addition, some genomic variants relevant to acid tolerance in Z. mobilis have been identified as well. For example, the acetate-tolerant phenotype in AcR mutant may be due to the over-expression of ZMO0119 encoding Na^+/H^+ antiporter resulting from a 1.5-kb deletion in AcR mutant [25, 26]. Recently, single nucleotide variants (SNVs) in genes ZMO0056 and ZMO0589, which encode a glutamine-fructose-

6-phosphate aminotransferase and a DNA repair protein RadA respectively, have been characterize to likely contribute to acid tolerance in mutant stains developed by a multi-round atmospheric and room temperature plasma (mARTP) mutagenesis [32]. However, it is still a challenge to develop strains tolerant to acidic pH due to the lack of a comprehensive understanding on functional genomics and molecular regulation responsible for the acid tolerance in Z. mobilis.

Adaptive laboratory evolution (ALE) is a powerful tool for strains development with desired stable phenotypes without requiring knowledge of any underlying genetic mechanism [33–35]. It has been successfully applied in model organisms, such as E. coli [36, 37] and Saccharomyces cerevisiae [38, 39]. After obtaining mutant strain, next generation sequencing (NGS) based strategies such as whole genome Resequencing (WGR) and RNA sequencing (RNA-Seq) are usually applied to reveal genetic changes and global gene expression changes [40]. Currently, ALE strategy has been employed for strain improvement in Z. mobilis, such as inhibitor tolerance [28, 29] and substrate utilization [41–43]. In this study, ALE was performed under acidic pH conditions to select Z. mobilis mutant strains with enhanced acidic pH tolerance. WGR and RNA-Seq technologies were then applied to investigate the genotypic changes associated with acidic-pH tolerabce revealing the molecular mechanism responsible for the improved acidic pH tolerance in mutant strains.

Results And Discussion

Development of acidic-pH tolerant mutants of *Z. mobilis* through adaptive laboratory evolution (ALE)

Z. mobilis was reported to be able to grow within a broad pH range (3.5-7.5) [22], the growth of *Z. mobilis* in pH range below pH 4.0 was further investigated in this study. The result showed that ZM4 can grow below pH 4.0 **(Fig. 1)**, which is consistent with previously reported [22, 44]. However, when the pH value decreased from 4.0 to 3.5, the lower of the medium pH was, the longer of lag phase took and the lower of biomass produced **(Fig. 1)**. Cells almost could not grow at pH below 3.5 **(Fig. 1)**, which might be ascribed to the damage of cell membrane structure and protein configuration at acidic pH [45]. Therefore, the development of an acidic pH tolerant strain could benefit directly in commercial bioethanol production under acidic fermentation conditions using lignocellulosic feedstock hydrolyzed by acid as the substrate.

Subsequently, adaptive evolution was carried out with two parallel experiments in RMG2, which firstly adapted at pH 4.0 with 30 cycles and then transferred to pH 3.5 and pH 3.6 for acidic pH evolution (**Fig. 2A**). Finally, after 55 cycles of cultivation at pH 3.5 and 75 cycles at pH 3.6, four evolved cultures with enhanced acidic pH tolerance were obtained, and named as 3.5M-1, 3.5M-2, 3.6M-1, and 3.6M-2, respectively. The stability of these four adapted cultures was then analyzed at pH 3.6 with three colonies of each as replicates (**Fig. 2B**). The results showed that the growth of replicates from 3.5M-1 and 3.6M-1 was more uniformed than that of 3.5M-2 and 3.6M-2. The growth of these four mutants were then compared with wild-type ZM4 under different pH conditions, including pH 3.5, pH 3.6, pH 4.0, and pH 6.0 **(Fig. 3)**.

Under pH 3.5 condition (**Fig. 3A)**, all four mutants had higher growth rates and final OD values than ZM4, in which the mutant 3.6M-1 exhibited the maximum cell growth, followed by 3.6M-2, 3.5M-2 and 3.5M-1 successively. Under pH 3.6 condition, the mutants still had higher growth rates and shorter time reaching stationary phase than ZM4 (**Fig. 3B**). The mutants had no obvious advantage than ZM4 at either pH 4.0 or pH 6.0 (**Fig. 3C, 3D**). These results suggested that all mutants had enhanced tolerance at lower acidic pH conditions. Two mutants 3.5M-1 and 3.6M-1 with the most growth difference from ZM4 among all mutants were selected and renamed as 3.5M and 3.6M correspondingly for further studies.

Evaluation of cell growth, glucose consumption, and ethanol production of mutant strains 3.5M and 3.6M at acidic and neutral pH conditions

Since the acidic pH condition affects fermentation, ethanol production and glucose consumption, two mutant strains 3.5M and 3.6M were investigated at acidic and neutral pH conditions of pH 3.8 and pH 6.2, respectively. Both mutants 3.5M and 3.6M exhibited better cell growth and faster ethanol production than wild-type ZM4 at acidic pH 3.8 (**Fig. 4A**, **4B**). The growth rate of 3.5M and 3.6M was 0.23 h^{-1} and 0.35 h^{-1} respectively, while that of ZM4 was only 0.14 h^{-1} (**Table 1**). Consistent with the fast cell growth, the fermentation time were reduced significantly from 22 h for ZM4 to 18 h and 13 h for 3.5M and 3.6M respectively, leading to the increase of ethanol productivity by 21.21% and 64.65% correspondingly (**Table 1; Fig. 4A**, **4B**).

Under neutral pH condition of pH 6.2, cell growth and ethanol production of 3.6M were similar to those of ZM4, but performed better than those of 3.5M (**Table 1; Fig. 4C**, **4D**). These results suggested that 3.5M and 3.6M possessed relatively fast glucose consumption and ethanol production at acidic condition, and 3.6M maintained similar capacities as ZM4 at neutral pH condition, which can be used to replace ZM4 as the biocatalyst for bioethanol production fermenting well in both acidic and neutral pH conditions (**Table 1; Fig. 4C**, **4D**).

Table 1. Fermentation performance of time to consume all glucose (Time), growth rate, as well as ethanol titer, yield, and productivity of the wild-type *Z. mobilis* ZM4 and mutant strains 3.5M and 3.6M in RMG5 at pH 3.8 and pH 6.2.

Condition & Strain	Glucose used ($g \cdot L^{-1}$)	Time (h)	Growth rate (h^{-1})	Titer ($g \cdot L^{-1}$)	Yield (%)	Productivity ($g \cdot L^{-1} \cdot h^{-1}$)
pH 3.8						
ZM4	44.95±0.12	22	0.14±0.01	21.74±0.43	94.55±1.89	0.99±0.02
3.5M	44.91±0.01	18	0.23±0.01	21.62±0.14	94.13±0.64	1.20±0.01
3.6M	44.98±0.00	13	0.35±0.01	21.22±0.28	92.24±1.22	1.63±0.02
pH 6.2						
ZM4	44.91±0.11	10	0.49±0.007	20.21±1.33	87.99±5.88	2.02±0.13
3.5M	44.97±0.00	12	0.39±0.005	20.05±0.82	87.19±3.56	1.67±0.07
3.6M	44.95±0.00	10	0.49±0.01	20.24±0.36	90.00±1.20	2.02±0.04

The underlying mechanism of acidic-pH tolerance through NGS-based genome resequencing and RNA-Seq

To illustrate the underlying genetic basis responsible for the enhanced acidic pH tolerance, samples of mutant and wild-type strains cultured at acidic pH 3.8 and neutral pH 6.2 were collected for WGR to determine the genetic changes in 3.5M and 3.6M using the genome of parental strain ZM4 as the reference (ATCC 31821) (GenBank accession No. of NZ_CP023715 for chromosome, and NZ_CP023716, NZ_CP023717, NZ_CP023718, NZ_CP023719 for four plasmids) [46]. RNA-Seq was also employed to explore the global transcriptional differences among these strains at acidic and neutral pH conditions.

The WGR result identified several single-nucleotide polymorphisms (SNPs) in the mutants. These SNPs included mutations located in chromosome, 7 SNPs in 3.5M and 5 SNPs in 3.6M, which were listed in **Table 2**. Among these mutations, 4 common SNPs were found in both mutants locating at the coding sequence (CDS) region of 4 genes: *ZMO0421* (Ala67Thr), *ZMO0712* (Gly539Asp), *ZMO1432* (Pro480Leu), and *ZMO1733* (Thr7Lys) respectively (**Fig. 5A**). These common mutations may contribute to the enhanced acidic-pH tolerance of mutant strains, while other unique mutations that are not shared by these strains may contribute to the unique phenotypic differences of these strains.

Table 2. Single-nucleotide polymorphisms (SNPs) in mutant strains 3.5M and 3.6M compared with wild-type ZM4. "+/−" indicates the presence/absence of variation, and the numbers in bracket after "+" represent the frequency (%) of the SNP. AA means amino acid.

Locus	Ref	SNP	AA change	3.5M	3.6M	ZM4	Gene	Product
424761	C	T	Ala67Thr	+ (99.76)	+ (98.86)	-	*ZMO0421* (*hisC2*)	Histidinol-phosphate aminotransferase HisC
711194	G	A	Gly539Asp	+ (99.24)	+ (99.73)	-	*ZMO0712* (*ppk*)	Polyphosphate kinase
1449594	G	A	Pro480Leu	+ (100.0)	+ (99.72)	-	*ZMO1432*	Membrane protein component of efflux system
1779278	C	A	Thr7Lys	+ (100.0)	+ (99.71)	-	*ZMO1733* (*oxyR*)	Transcriptional regulator OxyR
1306151	C	T	Trp485*	+ (99.50)	-	-	*ZMO1291*	Peptidase S10 serine carboxypeptidase
1701191	G	A	Leu77Phe	+ (99.08)	-	-	*ZMO1651* (*ptsP*)	Signal transduction protein
173653	T	C		+ (100.0)	-	-	Intergenic region	Between *ZMO0183* and *ZMO0184*
1451222	A	G		-	+ (100.0)	-	Intergenic region	Between *ZMO1432* and *ZMO1433*

Additionally, the differentially expressed genes (DEGs) were identified through analysis of variance (ANOVA) using strains and different conditions as variables. A total of 914 genes were identified by comparing any two conditions with p-value < 0.05 (**Table S1**). There has 267, 303, and 681 DEGs comparing acidic pH condition with neutral pH condition of 3.5M, 3.6M and wild-type ZM4 respectively (**Fig. S1A**). 304, 319 and 134 DEGs were also identified comparing 3.5M with ZM4, 3.6M with ZM4, and 3.5M with 3.6M at acidic pH respectively (**Fig. S1B**). The DEGs from comparison of same strain under different pH conditions or different strains under acidic pH condition were then further analyzed.

Association of genes with common changes in mutants with enhanced acidic-pH tolerance: A common mutation was found in gene *ZMO0421*, encoding histidinol-phosphate aminotransferase HisC2, which catalyzes the seventh step in the histidine biosynthesis pathway. Previous studies in *Z. mobilis* showed that HisC2 has broad substrate specificity and participates in transamination reactions for tyrosine and aromatic amino acid (phenylalanine) biosynthesis, which is essential in all studied organisms [47]. The mutation in *ZMO0421* (Ala67Thr) was located in the amino transfer domain (PF00155, 32-357 aa) catalyzing the transamination reaction, which probably can enhance the enzymatic activity efficiency in acidic pH condition although detailed experiment is needed in the future.

Another common mutation was found in *ppk* gene (*ZMO0712*), which encodes polyphosphate kinase that transfers the γ-Pi of ATP to form a long chain polyphosphate (polyP) reversibly [48]. Several biological functions have been identified for cellular polyP including buffering capacities for pH homeostasis, DNA damage repair, cell cycle, motility, and biofilm formation [49-51]. Studies in other bacteria showed that polyP was rapidly accumulated by PPK under environmental stresses including acidic conditions [52-54]. Our transcriptomic data indicated that the expression of *ppk* in wild-type ZM4 was upregulated under acidic pH compared with neutral pH (**Table S2**), which is consistent with above reported conclusion. However, this gene was not significantly differentially expressed in mutant strains under acidic pH condition. Considering that the mutation in *ppk* (Gly539Asp) was located in the C2 domain (PF13090, 503-687), which is highly conserved in the PPK family and essential for the enzymatic activity [54], the mutation in this enzyme may help improve the activity of PPK resulting in the acceleration of polyP production to respond to the toxic acidic condition.

Additionally, mutation in gene *ZMO1432*, encoding the inner membrane component protein of a RND efflux system containing 12 transmembrane domains [55], was observed in both mutants. The mutation (Pro480Leu) was located at the eleventh transmembrane (TM11) domain, which may play an important role in the process of substrates extrusion from cytoplasm to periplasm by proton-motive force (PMF) with the conformational changes of RND system [55]. According to the prediction by TMHMM Server v. 2.0 [56], the transmembrane probability of TM11 domain in the mutant protein was improved from 0.7 to 1.0 (**Fig. S2**). Therefore, the mutation in *ZMO1432* (Pro480Leu) may increase the stability and rigidity of TM11 and hence indirectly improve the efficiency to resist acidic stress by pumping toxic substances out, such as organic acids or anions [57].

Moreover, a mutation (A to G) was also found in the intergenic region between *ZMO1432* and *ZMO1433* in mutant 3.6M (**Table 2**), which is in the upstream of the promoter region of *ZMO1432* predicted by BPROM [58]. As shown in the RNA-Seq result, the expression of the whole operon encoding a tripartite RND efflux system consisted of *ZMO1432*, *ZMO1431*, *ZMO1430* and *ZMO1429* was significantly upregulated at acidic pH in two mutant strains compared with ZM4, and 3.6M had the highest expression level among these strains (**Fig. 5E**). The mutation in the intergenic region in mutant 3.6M could help upregulate the expression of downstream genes, since the expression of these genes was also upregulated under pH 6.2 in 3.6M compared with ZM4 (**Table S2**). Combining these mutations and

transcriptomic results, the RND efflux pump may play a crucial role on acidic-pH resistance in mutant strains.

The last common mutation shared in both mutant strains was within *oxyR* gene (*ZMO1733*). OxyR is a LysR family transcriptional regulator consisting of an N-terminal DNA-binding domain (DBD) and a C-terminal regulatory domain (RD), which controls the OxyR regulon consisting of almost 40 genes that can help protect cells from oxidative stress [59]. The mutation in OxyR (Thr7Lys) was in the N-terminal of LysR-type helix-turn-helix (HTH) DNA-binding domain (PS50931, 6-63 aa), which likely changes the binding affinity of HTH with its target DNA sequence due to the amino acid change from threonine with short side chain to lysine with long side chain (**Table 2**). Our RNA-Seq results showed that several genes involved in reactive oxygen species (ROS) detoxification possibly regulated by OxyR, such as *ZMO0918* (catalase), *ZMO1060* (superoxide dismutase), *ZMO1732* (alkyl hydroperoxide reductase), and *ZMO1211* (glutathione reductase), were significantly upregulated in all strains, especially in ZM4 at acidic pH compared with neutral pH condition (**Table S2**). Since acidic pH could induce a secondary oxidative stress and the acid tolerance response overlaps with the oxidative stress response [60, 61], the mutation in *oxyR* could contribute to the acidic pH tolerance in mutant strains.

Since mutant strains with these mutations exhibited advantages under acidic pH condition compared with wild-type ZM4, these mutations could be crucial for *Z. mobilis* to resist the acidic pH stress although further investigation is needed to help confirm whether they are necessary for the acidic-pH resistance phenotype and how.

Upregulation of genes associated with membrane components for enhanced acidic-pH tolerance: The lipid composition of cell membrane could be reconfigured at acidic pH condition, which will affect proton permeability directly or indirectly [62]. Hopanoids, one of the outer membrane component in Gram-negative bacteria such as *Z. mobilis* playing a role in regulating the fluidity and permeability of membrane [63, 64], are also contributed to the high ethanol tolerance of *Z. mobilis* [65]. It was reported that the deletion of hopanoid biosynthesis associated gene *shc* (*ZMO0872* in ZM4), encoding the squalene-hopene cyclase protein, led the mutant more sensitive to acidic pH than the wild-type strains of *Burkholderia cenocepacia* or *Rhodopseudomonas palustris* TIE-1 [64, 66]. Our RNA-Seq data indicated that most hopanoid biosynthesis associated genes (*ZMO0867*, *ZMO0872*, *ZMO0873*, *ZMO0874*) were significantly downregulated in ZM4 at acidic pH compared with those at neutral pH, but not in mutant strains (**Fig. 5B; Table S2**), which suggested that mutant strains may have relative higher hopanoid expression than ZM4 at acidic pH. Notably, gene *ZMO0867*, encoding hopanoid-associated sugar epimerase HpnA protein, is a member of H^+ and NADPH-consuming protein (EC1.1.1.219). Compared with ZM4, the upregulation of hopanoid biosynthesis gens such as *hpnA* (*ZMO0876*) in two mutant strains under acidic pH condition (**Fig. 5B; Table S2**) suggested that mutant strains may consume more cytoplasmic protons during hopanoid biosynthesis resulting in higher content of hopanoids and less cytoplasmic protons than ZM4 for enhanced acidic-pH tolerance.

The modification of the phospholipids in the inner membrane is also a strategy to reduce proton permeability. In many bacteria, the resistance to acidic pH is associated with the conversion of unsaturated fatty acids (UFAs) into cyclopropane fatty acids (CFAs) through the addition of a methyl group to the double bond of UFA, which is associated with cyclopropane fatty acid synthase (Cfa). The expression of *cfa* gene is usually upregulated under acidic conditions [67, 68], and a similar up-regulation was observed for gene *ZMO1033* encoding Cfa in ZM4 at acidic pH, which suggested that *cfa* gene may be associated with outer membrane modification and acidic-pH tolerance (**Fig. 5B; Table S2**).

Energy generation through increased glycolysis and energy conservation through decreased cell growth and motility for acidic-pH resistance: It is reported that *Streptococcus mutans* altered its metabolism by increasing the glycolytic activity to produce ATP at acidic pH condition [69, 70], and ATP utilization was further derived from cell growth for acid tolerance [71]. Although ED pathway only produces one mole ATP per single mole glucose, it is reported that ED pathway in *Z. mobilis* is nearly twice as thermodynamically favorable as EMP pathway in *E. coli* or *S. cerevisiae* [72]. Our RNA-Seq result demonstrated that 6 genes involved in glycolysis pathway (*ZMO1478 (pgl), ZMO0997 (eda), ZMO0177 (gap), ZMO1240 (gpmA), ZMO1608 (eno)* and *ZMO0152 (pyk)*) were significantly upregulated at acidic pH compared with neutral pH in ZM4 and 3.6M, which could help produce more ATP for acidic pH tolerance (**Fig. 5C; Table S2**). Correspondingly, the final $\log_2$OD values of three strains at pH 3.8 were lower than those at pH 6.2, which was about 1.90 and 2.34, respectively (**Fig. 4A, 4C**), indicating more energy was used for acidic pH resistance instead of cell growth. This energy-demanding process might explain the uncoupling between glycolytic and biosynthetic reaction in *Z. mobilis* [73] with more energy consumed for acidic pH resistance. Additionally, gene *ZMO1754* encoding SsdA that catalyzes the production of acetate from acetaldehyde was upregulated significantly at pH 3.8 compared with pH 6.2 for both mutants and especially wild-typeZM4, which was however significantly downregulated at pH 3.8 in mutant background compared with ZM4 (**Fig. 5C; Table S2**). These results indicated that more acetate might be produced at acidic pH than at neutral pH condition, and mutants produced less protonated acetate and consumed less NAD^+ for a more efficient glycolysis and less acidified cytoplasmic environment than wild-type ZM4.

In addition, a number of genes encoding flagellar structure proteins and chemotaxis related proteins were significantly downregulated under acidic pH condition compared with neutral pH condition in both ZM4 and mutant strains (**Fig. 5D; Table S2**), which could also help save energy for survival in the acidic-pH condition [74]. Therefore, the conservation of energy from cell growth and cell motility may help re-distribute the cellular energy for inhibitory acidic-pH resistance in *Z. mobilis*.

Upregulation of transporter and efflux pump helped maintain pH homeostasis in acidic conditions: In acidic pH conditions (pH < 4.76), some protonated acids such as acetic acid enter the cell through the Fps1p channel or by diffusion in uncharged state, which will then be dissociated in cytoplasm with a neutral internal pH environment, and then affect cell metabolism and growth [75, 76]. HCl, which used in this study for pH adjustment, could enter the cytoplasm in undissociated state [75] at acidic pH, and then dissociate into H^+ and Cl^- in the cytoplasm. The accumulation of the H^+ could activate cellular acid

resistant system, while the accumulation of Cl^- could change the membrane potential. It was reported that the CLC Cl^-/H^+ exchangers control the homeostasis of cellular compartments in most living organisms by catalyzing the exchange of two Cl^- for one H^+ in opposite direction [77, 78]. Our result indicated that the transcriptional level of CLC H^+/Cl^- antiporter, encoded by *ZMO0841*, was significantly downregulated in ZM4 under acidic pH, but upregulated at acidic pH in 3.6M compared with ZM4 (**Fig. 5E; Table S2**). These results suggested that CLC transporter may play an important role in homeostasis by controlling the membrane potential under acidic pH conditions, especially for the enhanced acidic-pH resistance capability of mutant strain 3.6M.

The increase of glycolysis pathway activity at acidic pH suggested that the accumulation of acidic end-products such as acetate and lactate could lead to an acidic intracellular condition [69]. Therefore, it is important for cell to export acidic products to maintain intracellular homeostasis. ATP-binding cassette (ABC) transporters transport a wide spectrum of substrates from small inorganic and organic molecules to larger organic compounds [79], and have been confirmed to contribute to acetic acid tolerance as an efflux pump of acetic acid [79]. Our results demonstrated that five genes encoding ABC transporters (*ZMO0143, ZMO1017, ZMO0799-ZMO0801*) were significantly upregulated in both mutant strains compared with ZM4 at acidic pH condition (**Fig. 5E**; **Table S2**). Moreover, RND efflux pump is well-known for transporting various compounds including cationic dyes, antibiotics, detergents and even simple organic solvents with the proton antiport [57, 80, 81]. Our result indicated that an RND efflux pump encoded by *ZMO1429-ZMO1432* was also significantly upregulated at acidic pH in both mutant strains compared with ZM4. The upregulation of ABC transporter and efflux pump may suggest an enhanced capability of mutant strains to maintain cytoplasmic homeostasis in acidic pH conditions.

In addition, pumping H^+ out of the cytoplasm is another efficient way in the maintenance of pH homeostasis [82]. F_1F_o ATP synthase (F_1F_o ATPase) can utilize the proton gradient for ATP synthesis, it can also reverse and hydrolyze ATP to pump H^+ out to maintain the intracellular pH homeostasis [83, 84]. For example, genes encoding F_1F_o ATPase in *Streptococcus mutans* was upregulated at acidic pH to help resist acid stress [85]. Another work indicated that when respiration was impeded, F_1F_o ATPase hydrolyzed ATP to pump protons and contributed to intracellular neutral condition maintaining the essential mitochondrial membrane potential [86]. Our results demonstrated that 7 genes encoding F_1F_o ATP synthase (*ZMO0239*, *ZMO0240*, *ZMO0241*, *ZMO0667*, *ZMO0668*, *ZMO0669*, *ZMO0671*) and another gene encoding F_1F_o ATP synthase assembly protein (*ZMO2005*) were significantly upregulated at acidic pH compared with neutral pH for mutant strain 3.6M (**Fig. 5F; Table S2**). Since cellular respiration process was uncoupled with cell growth in *Z. mobilis* [87], and the ATP generation was majorly from glycolysis whose activity was increased as discussed above, the upregulation of F_1F_o ATPase genes may possibly help pump H^+ out from the cytoplasm through consuming ATP.

Furthermore, proton translocation was suggested to result in an alkalization of the intracellular medium in *Z. mobilis* at pH 6.5 during the respiration by transferring the H^+ out of cytoplasm [88]. Nine genes related to respiration chain for transferring electrons to oxygen, *ZMO0012, ZMO0568, ZMO0956-*

ZMO0958, ZMO1253-ZMO1255, and *ZMO1258*, were significantly downregulated in ZM4 at acidic pH compared with neutral pH (**Fig. 5F; Table S2**). In addition, six genes (*ZMO1809-ZMO1814*) encoding Rnf complex and an assembly gene (*ZMO1808*) were also downregulated at acidic pH compared with neutral pH in ZM4 but not in mutant strains (**Fig. 5F; Table S2**). The Rnf complex is required for electron transfer to nitrogenase during nitrogen fixation with proton excretion in *Rhodobacter capsulatus* [89]. Furthermore, gene *ZMO0456* encoding the ferredoxin which is the electron acceptor from NADH and electron donor for nitrogenase was also downregulated at acidic pH compared with neutral pH in ZM4 (**Fig. 5F; Table S2**). The downregulation of genes associated with electron transfer chain at acidic pH condition in wild-type ZM4 could make the excretion of protons against proton gradient from cytoplasm difficult, leading to growth inhibition. In contrast, the expression of these genes in mutant background was not significantly downregulated in acidic pH compared with neutral pH condition. Instead, they were upregulated compared with ZM4 in acidic pH condition (**Fig. 5F; Table S2**). These results indicated that mutants could maintain relatively high proton transportation capacity against acidic pH condition.

Proton consumption and alkaline compound production for enhanced acidic-pH resistance: Biosynthesis of branched-chain amino acids (BCAAs) was reported to reduce H^+ concentration in the cytoplasm by consuming proton or producing ammonia [69]. Two genes involved in the conversion of isoleucine from threonine in *Z. mobilis* (*ZMO0687* and *ZMO1275*) were significantly up-regulated under acidic pH in mutant 3.6M compared with ZM4 (**Fig. 5G; Table S2**). *ZMO0687*, encoding acetolactate synthase large subunit, participates in the process with proton consumption in the first step. And *ZMO1275,* encoding threonine dehydratase, is involved in the process with ammonia production in the second step. Meanwhile, the fourth step of this conversion was catalyzed by dihydroxy-aid dehydratase encoded by *ZMO1792,* which had a reduced expression level at acidic pH compared with neutral pH only in wild type (**Fig. 5G; Table S2**). Moreover, genes *ZMO0687* and *ZMO1792* are also involved in the first step with proton consumption and the third step of valine biosynthesis from pyruvate. Interestingly, it was reported that the deviation of pyruvate away from lactic acid and acetate production toward other metabolic pathways was effective toward acidic end-product modulation in *S. mutans* [90]. The deviation of pyruvate from acidic production in mutants especially 3.6M was consistent with the result of less acetate production at acidic pH condition in mutants than ZM4 as described above. These changes indicated that 3.6M possesses higher activity on the conversion of BCAAs from threonine with more proton consumption and ammonia production than ZM4 and 3.5M.

In addition, gene *ZMO0296* encoding adenosine deaminase (Ada) to convert adenosine into inosine with ammonia production was significantly upregulated at acidic pH in both mutant strains compared with ZM4 (**Fig. 5G; Table S2**). Furthermore, the expression of *ZMO1207* gene encoding nitrilase (Nit, EC 3.5.5.1) that catalyzes the substrate containing cyanogroup to ammonia was also upregulated in mutant strain 3.6M only at acidic pH condition (**Fig. 5G; Table S2**). At acidic pH condition, ammonia could react with proton to produce the ammonium ion [91], which indicated that mutant strains possess greater capacity than ZM4 to neutralize the intracellular pH by proton-consuming and alkali-producing reactions resulting in enhanced acidic pH resistance.

However, the cytoplasmic pH homeostasis is connected with the proton motive force (PMF), which consists of two components of a transmembrane pH gradient (ΔpH) and a transmembrane electrical potential (Δψ) maintaining intercellular negative relative to outside [91]. The production of NH_4^+ from NH_3 and proton would reduce the ΔpH, but would result in excess intracellular positive charges, which would lead to a positive internal Δψ at any time that could destroy the PMF and impair cellular functions. To balance the excess intracellular positive charges, exporting NH_3 and NH_4^+ by ammonium transporter would avoid excessive positive charges hyperpolarizing the cell membrane [91]. our RNA-Seq result showed that the transcriptional level of ammonium transporter encoded by *ZMO0346* was upregulated significantly at acidic pH compared with neutral pH in both mutant strains (**Fig. 5G; Table S2**), which ensures the normal PMF function on the membrane. Moreover, it was reported that the conversion of CO_2 to HCO_3^- by carbonate anhydrase (CA) also contributed to acid-base equilibrium in *H. pylori* [22, 91]. It is interesting that the transcriptional level of *ZMO1133* encoding carbonate anhydrase was significantly upregulated at acidic pH compared with neutral pH in all strains (**Fig. 5G; Table S2**). Since *Z. mobilis* can consume sugars and produce CO_2 efficiently [92], CO_2/HCO_3^- could also be involved in keeping acid-base equilibrium at acidic pH conditions.

Reduced energy consumption on macromolecular repair for enhanced acidic-pH tolerance of mutant strains: Cell membrane, proteins and DNA would be damaged when bacteria were cultured in acidic environments. To reduce the damage, the expression of repair and defense proteins such as DnaK, RecA, UvrA, IrrE and AP endonuclease would be increased to protect the macromolecules from the damage [69, 84]. Our result showed that the transcription level of *ZMO0660* (*dnaK*) together with its co-chaperone *ZMO1690* (*dnaJ*) and regulator *ZMO0016* (*grpE*), and *ZMO1588* (*uvrA*) with its subunit *ZMO0362* (*uvrB*) were upregulated in ZM4 at acidic pH than neutral pH (**Fig. 5H; Table S2**), which demonstrated that it is necessary to enhance the expression of these proteins in order to protect DNA and protein from damage in acidic cytoplasm.

However, the expression level of these proteins was down-regulated at acidic pH in mutants compared with wild-type ZM4, except for gene *recA* that had no significant changes at different pH conditions in any strains. In addition, the transcriptional level of *ZMO1929*, encoding protein GroEL, which was important during adaptation to acid [69], was downregulated at acidic pH in mutant strains compared with wild type (**Fig. 5H; Table S2**). The deficient in HtrA, a surface protease involved in the degradation of aberrant proteins, reduced the ability of the mutant strain to endure acidic conditions [93], which demonstrated that this protein is important for cell to defense acid conditions. The transcriptional level of *ZMO0015*, encoding transcription repressor HrcA, was also upregulated significantly at acidic pH compared with neutral pH in wild type, and downregulated at acidic pH in mutant strains compared with wild type (**Fig. 5H; Table S2**). Moreover, the expression level of Clp protease complex, *ZMO0948* (*clpP*), *ZMO0949* (*clpX*), *ZMO0405* (*clpA*) and *ZMO1424* (*clpB*), which is involved in the remodeling and reactivation activities of proteins [69, 94], altered as same as *ZMO1929* and *ZMO0015*. The phenomenon that the expression level of macromolecular repair proteins which are indispensable for acid resistance were upregulated at acidic pH only in wild type or downregulated significantly in both mutant strains compared with wild type at

acidic pH, indicated that great demand on these proteins is needed for wild-type ZM4 to survive in acidic pH conditions, while mutants managed to avoid triggering defense responses and thus conserved energy used for macromolecule repair in ZM4.

Genetics confirmation of genes associated with acidic-pH resistance in *Z. mobilis* ZM4

To evaluate the impact of candidate genes associated with acidic-pH resistance identified through our genomics and transcriptomics study as discussed above, six plasmids containing candidate operons were constructed based on the shuttle vector pEZ15Asp with P*tet* as the promoter [95]. These candidate operons including *ZMO0142-ZMO0145* encoding ABC transporter, *ZMO0798-ZMO0801* encoding multiple drug efflux, *ZMO0956-ZMO0958* encoding cytochrome bc1 complex, *ZMO0238-ZMO0242* encoding ATP synthesis F_1 submits, *ZMO1428-ZMO1432* encoding RND efflux system with a mutation in *ZMO1432,* and *ZMO2005, ZMO0667-ZMO0671* encoding ATP synthesis F_0 submits were cloned into pEZ15Asp shuttle vector, which were named pEZ-Tc1, pEZ-Tc2, pEZ-Tc3, pEZ-Tc4, pEZ-Tc5(M) and pEZ-Tc6, respectively. These plasmid constructs including the empty vector pEZ15Asp as the control were then introduced into ZM4 separately. These recombinant strains were then investigated under different conditions to examine their impact on cell growth (**Fig. 6, Fig. S2**).

With the increase of tetracycline inducer concentration from 0 to 0.8 μg/mL, the growth advantage of recombinant strain containing pEZ-Tc3 decreased in acidic pH condition (**Fig. 6A**, **6B**, **6C**). Our previous work demonstrated that *Ptet* promoter driven the operon expression used in this study is leaky even when tetracycline was not supplemented into the medium [95, 96]. Therefore, this result suggested that the cytochrome bc1 complex encoded by the operon *ZMO0956-ZMO0958* in the recombinant strain containing pEZ-Tc3 could contribute to the acidic pH tolerance in *Z. mobilis* at a low expression level, which is consistent with our RNA-Seq result that the reduced expression of genes associated with electron transfer chain impacted the acid resistance of wild-type ZM4 (**Fig. 5F; Table S2**). On the contrary, with the increase of tetracycline inducer concentration from 0 to 0.8 μg/mL, the growth advantage of recombinant strain containing pEZ-Tc5(M) increased in acidic pH condition (**Fig. 6D**, **6E**, **6F**), which is again consistent with the significant upregulation of these genes in our RNA-Seq study (**Fig. 5E**; **Table S2**). Although further investigation is still needed to understand the association of acidic pH resistance with the different expression levels of these genes, our result suggested that the mutation in the intergenic region of upstream of *ZMO1432* in mutant 3.6M may contribute to the upregulation of downstream gene, and a higher expression of RND efflux pump is more advantageous for strain to defend acidic pH condition.

Recombinant strains containing other four operons had no advantageous effect for cell growth in acidic pH condition with or without tetracycline induction (**Fig. S3**). Instead, the growth of recombinant strain containing pEZ-Tc2 dramatically impeded when induced with 0.4 μg/mL tetracycline (**Fig. S3C, S3D**), and the growth of recombinant strain containing pEZ-Tc6 was inhibited with or without the supplementation of tetracycline inducer (**Fig. S3G, S2H**). Since these operons encode ABC transporter, multiple drug efflux, and ATP synthase submits, which may function with other cellular component coordinately, a delicate

balance of these operons with other genes may be needed for acidic pH resistance similar to previous report that the tailored expression of multiple genes simultaneously was essential for enhanced low-pH tolerance in *E. coli* [97].

Conclusion

Two acidic-pH tolerant mutants 3.6M and 3.5M of Z. mobilis were obtained from wild-type ZM4 by ALE strategy in this study, which possessed advantages at acidic pH conditions including high growth rate and ethanol productivity. Genetic changes and gene expression at different pH conditions were investigated using NGS-based genome resequencing and RNA-Seq with the underlying mechanism of acidic pH resistance proposed. Specifically, Z. mobilis altered its metabolic flux through genomic change affecting gene and gene expression associated with membrane composition and structure, proton transportation, energy conservation and redistribution for acidic pH resistance. Mutant strains had genes differentially expressed at acidic pH conditions to help modulate the membrane composition, strengthen membrane-associated transporters and electron transport system, and increase proton consumption and alkaline metabolite production to maintain proton permeability and cellular homeostasis. In addition, the enhanced branch amino acid biosynthesis and F_1F_o ATPase were also upregulated in the mutant 3.6M, which could contribute to its advantage in acidic pH condition over another mutant 3.5M and wild-type ZM4. Genetics study results demonstrated that the introduction of plasmid constructs containing operons expressing cytochrome bc1 complex or RND efflux pump could help Z. mobilis tolerate acidic pH conditions. This study not only obtained and characterized two acidic pH resistant mutant strains of Z. mobilis, which can be used as candidate strains for commercial bioethanol production under acidic fermentation conditions. In addition, molecular mechanism of acidic pH tolerance of Z. mobilis proposed in this study can also facilitate future research on rational design of synthetic microorganisms with enhanced tolerance against acidic pH conditions, and the strategy we developed in this study combining ALE, genome resequencing, RNA-Seq and classical genetics study for mutant evolution and characterization can be applied in other industrial microorganisms.

Materials And Methods

Bacterial strain and culture conditions

Escherichia coli DH5α from Invitrogen (USA), was cultured in Lysogeny Broth (LB, 10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract, and 1.5% agar for solid). *Z. mobilis* ZM4 (ATCC 31821) was cultured in Rich Medium (RM, 10 g/L yeast extract, 2 g/L KH_2PO_4, different concentration of glucose as required, and 1.5% agar for solid) at 30 °C with sharking at 100 rpm. pH was adjusted using HCl and KOH. For pH tolerance tests, glucose concentration in the media was 20.0 g/L (RMG2) and 50.0 g/L (RMG5).

Adaptive laboratory evolution

Adaptive laboratory evolution was applied to evolve the wild-type strain *Z. mobilis* ZM4 (**Fig. 2A**). Cells were firstly streaked on RMG2 plate from stock, two colonies were then selected randomly and cultivated in liquid RMG2. After grown to the mid-exponential phase (OD_{600nm} = 0.80-2.50, determined by spectrophotometer [UV-1800, AOE, Chain]), cells were then transferred into fresh RMG2 at pH 4.0 with the initial OD_{600nm} value of 0.1. When reaching to the mid-exponential phase, strains were transferred again into the same medium. After 30 cycles of cultivation at pH 4.0, the adapted strains were further transferred to fresh medium at pH 3.5 or pH 3.6. Finally, four evolved strains with enhanced tolerance to acidic pH were obtained, two adapted with 55 cycles of cultivation at pH 3.5 and other two with 75 cycles of cultivation at pH 3.6 (**Fig. 2A**).

Stability and pH tolerance evaluation of evolved cultures using Bioscreen C

Four evolved cultures were streaked on RMG2 plate, three colonies were then selected randomly and cultured overnight in 2 mL liquid RMG2 to the exponential phase. Seed cultures were washed twice and transferred into different media with an initial OD_{600nm} value of 0.1. Media were adjusted to pH 3.6 for the stability analysis, or adjusted to pH 3.5, pH 3.6, pH 4.0, and pH 6.0 respectively for the pH tolerance analysis. Cell growth was monitored at 600 nm using a Bioscreen C instrument (Growth Curves USA, NJ) with three technical replicates [98]. The working volume was 200 µL/well. Temperature was controlled at 30 °C and the absorbance values were automatically read at regular intervals of 15 min. The experiments were repeated at least twice.

Cell growth and fermentation analysis

Fermentations were performed in 500 mL shake flasks with 400 mL RMG5 at 30 °C with an agitation rate of 100 rpm, and the wild-type ZM4 was used as the control. The pH of the media was adjusted at 3.8 and 6.2 by automatic titration with HCl and KOH. During the fermentation, Cell growth was determined every 2 h by measuring the optical density at 600 nm (OD_{600}) using spectrophotometer (UV-1800, AOE, Chain). The samples were also collected and centrifuged at 10,000 rpm for 5 min, and filtered through 0.22 µm filters. Samples at exponential phase were harvested for genome resequencing and RNA-Seq. Glucose and ethanol in filtered supernatants were analyzed using high-pressure liquid chromatography (HPLC, LC-20 AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 60 °C at the flow rate of 0.5 mL/min using 5 mM H_2SO_4 as the mobile phase.

Whole genome resequencing analysis

Whole-genome resequencing were performed using the paired-end sequencing technology according to standard Illumina protocols by IgeneCode, Inc., Beijing, China. The paired-end reads quality was checked using FastQC program (http://www.bioinformatics.babra ham.ac.uk/projects/fastqc/). Data passing the quality control were then mapped to the reference genome sequences of *Z. mobilis* ZM4, ATCC 31821 (GenBank accession No. of chromosome: NZ_CP023715, and plasmids: NZ_CP023716, NZ_CP023717, NZ_CP023718, NZ_CP023719) using the CLC Genomics Workbench (version 11.0) to identify the

genomic variations. The objective mutations of the mutant strains were obtained with the parental wild-type strain as control, of which the mutation frequency more than 30% was filtered.

RNA-Seq Transcriptomic analysis

Transcriptomics study was followed previous work [46, 99]. Briefly, cell cultures were collected at the log phase under different pH conditions. RNA-Seq was performed using the same strategy as genome resequencing mentioned before. Data passing the quality control were imported into CLC Genomics Workbench (version 11.0) for RNA-Seq analysis to get the RPKM values with *Z. mobilis* ZM4, ATCC 31821 (GenBank accession No. of chromosome: NZ_CP023715, and plasmids: NZ_CP023716, NZ_CP023717, NZ_CP023718, NZ_CP023719) and four related plasmids as the reference genome. Gene expression normalization, analysis of variance (ANOVA), and hierarchical clustering analysis were conducted using JMP Genomics (version 9.0) to identify differentially expressed genes at different conditions. Significantly differential expression genes were determined with a selection threshold of *P*-value ≤ 0.01 and $\log_2$-fold change ≥1 (significant induction) or ≤ -1 (significant repression). Duplicate samples were used for each condition.

Genetics study evaluating the impact of candidate operons on acidic pH tolerance

Six operon candidates, *ZMO0142-ZMO0145*, *ZMO0798-ZMO0801, ZMO0956-ZMO0958, ZMO0238-ZMO0242, ZMO1429-ZMO1432, ZMO2005* with *ZMO0667-ZMO0671,* encoding ABC transporter related protein, multiple drug efflux, cytochrome bc1 complex, ATP synthesis F_1 submits, RND efflux pump, ATP synthesis F_0 submits, were amplified from the genomic DNA of *Z. mobilis* except that *ZMO1429-ZMO1432* was amplified from the genomic DNA of mutant strain with a mutation on the gene *ZM01432* using primers listed in **Table S3.** The PCR products were then cloned into the pEZ15Asp shutter vector with P*tet* as the promoter [95] by the Gibson assembly method [100]. Each primer for operon amplification contains about 15-20 nucleotide overlapping region of vector. Recombinant strains containing correct plasmid construct were identified by colony PCR, and confirmed by Sanger sequencing (Tsingke, China). These plasmids were named as pEZ-Tc1, pEZ-Tc2, pEZ-Tc3, pEZ-Tc4, pEZ-Tc5(M) and pEZ-Tc6, respectively.

The correct plasmids were then transformed into *Z. mobilis* competent cells via electroporation (0.1-cm electrode gap, 1600 V, 200 Ω, 25 μF) using a Gene Pulser® (Bio-Rad, USA) as described previously [101]. Candidate strains containing correct plasmid construct were identified by colony PCR, and confirmed by Sanger sequencing (Tsingke, China). Cell growth of these strains was evaluated at different pH (3.6, 4.0, 6.0) in RMG5 medium using Bioscreen C. Tetracycline was added at concentrations of 0.4 μg/mL and 0.8 μg/mL to induce genes expression.

Abbreviations

ABC: ATP-binding Cassette; **ADA:** Adenosine deaminase; **ALE:** Adaptive Laboratory Evolution; **ANOVA**: Analysis of Variance; **BCAAs:** Branched-chain Amino Acids; **CA:** Carbonate Dehydratase; **CDS:** Coding

sequence; **CFAs:** Cyclopropane Fatty Acids; **DBD:** DNA-binding Domain; **DEGs:** Expressed genes; **GRAS:** Generally Regarded as Safe; **HPLC:** High-Pressure Liquid Chromatography; **HTH:** Helix-Turn-Helix; **LB**: Lysogeny Broth; **mARTP:** Multi-round Atmospheric and Room Temperature Plasma; **NGS:** Next-generation Sequencing; **NIT:** nitrilase; **NTG:** Nitrosoguanidine; **PCR:** Polymerase Chain Reaction; **PMF:** Proton Motive Force; **polyP:** Polyphosphate; **RD:** Regulatory Domain; **RM**: Rich Medium; **RNA-Seq:** RNA Sequencing; **ROS**: Reactive Oxygen Species; **SNPs:** Single-nucleotide Polymorphisms; **UFAs:** Unsaturated Fatty Acids; **WGR:** Whole Genome Resequencing; **ΔpH:** pH Gradient; **Δψ:** Transmembrane Electrical Potential.

Declarations

Ethics approval and consent to participate

The authors declare that this study does not involve human subjects, human material and human data.

Consent for publication

All authors read and approved the final manuscript.

Availability of data and material

The authors declare that all data supporting the findings of this study are available within the paper and its Supplemental files or from the corresponding author on request. The raw data of genome resequencing and RNA-Seq were deposited into Sequence Read Archive (SRA) database with the BioProject accession numbers of PRJNA590883[1] and PRJNA553033[2], respectively.

Competing Interests

The authors declare that they have a patent application associated with mutant strains developed in this study.

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Authors' contributions

SY conceived and designed the experiments with inputs from all authors. QY evolved strains and tested the fermentation with the help from SW, YT, YZ and JG. YY handled and analyzed the high-throughput sequencing data. QY, YY, WX, YC and SY prepared figures and tables, and wrote the manuscript. QY, YY, XW, YC, MH, BW, SC and SY discussed and revised the manuscript. All authors contributed to data analyses, read, revised and approved the final manuscript.

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[1] https://dataview.ncbi.nlm.nih.gov/object/PRJNA590883?reviewer=b5on485mgfrk0e5qs37loi10eh

[2] https://dataview.ncbi.nlm.nih.gov/object/PRJNA553033?reviewer=uphnpo9ouki2purbjco8p9m2f3

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Figures

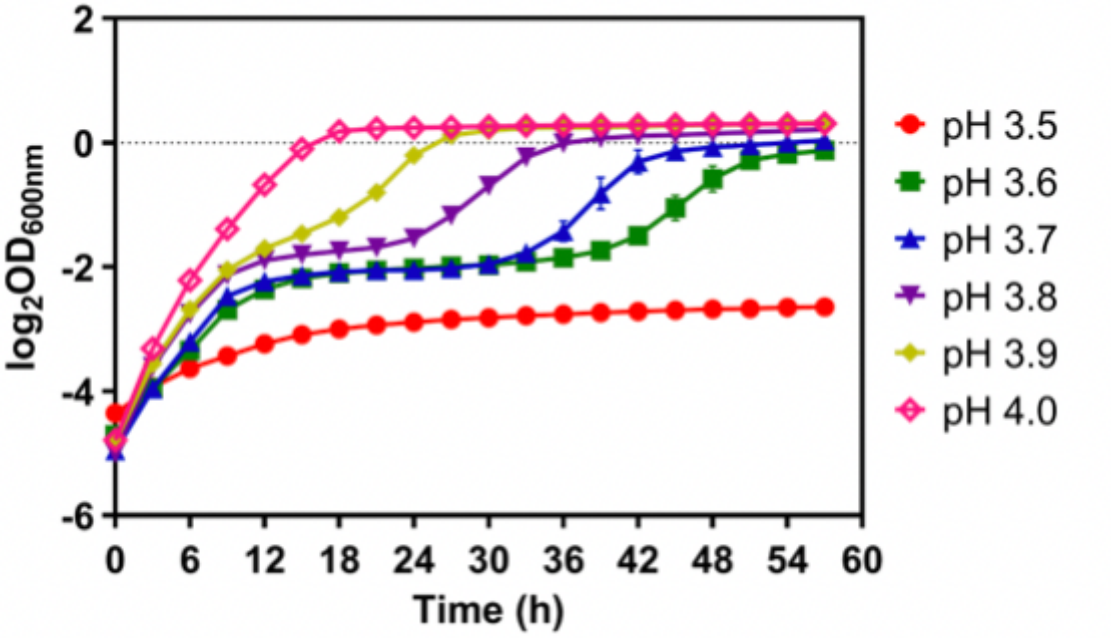

Figure 1

Cell growth of wild-type Z. mobilis ZM4 under different pH conditions. ZM4 was cultured in RMG5 using Bioscreen C instrument at a pH range within pH 3.5-4.0. At least two independent experiments were performed with similar results. Values are the mean of one representative experiment with three technical replicates. Error bars represent standard deviations.

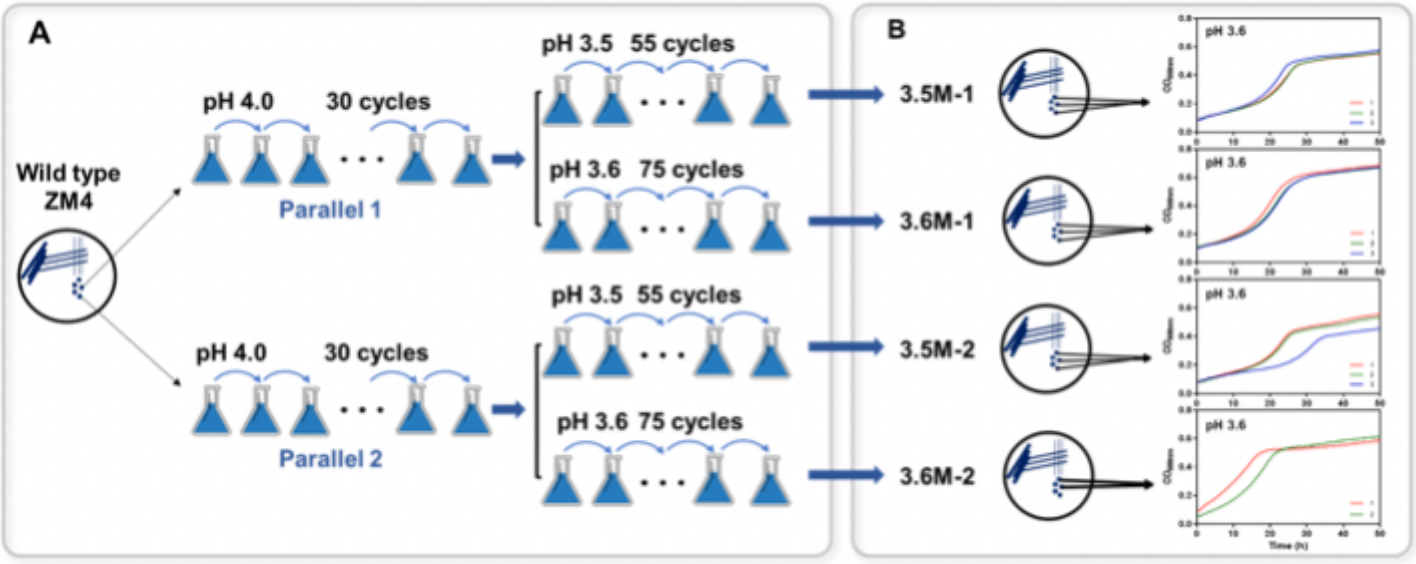

Figure 2

The workflow to obtain acidic-pH resistant mutants of Z. mobilis ZM4 through adaptive laboratory evolution (ALE) in RMG2 media (A), and the verification and selection of mutants with stable acidic-pH

resistance under pH 3.6 culture condition (B).

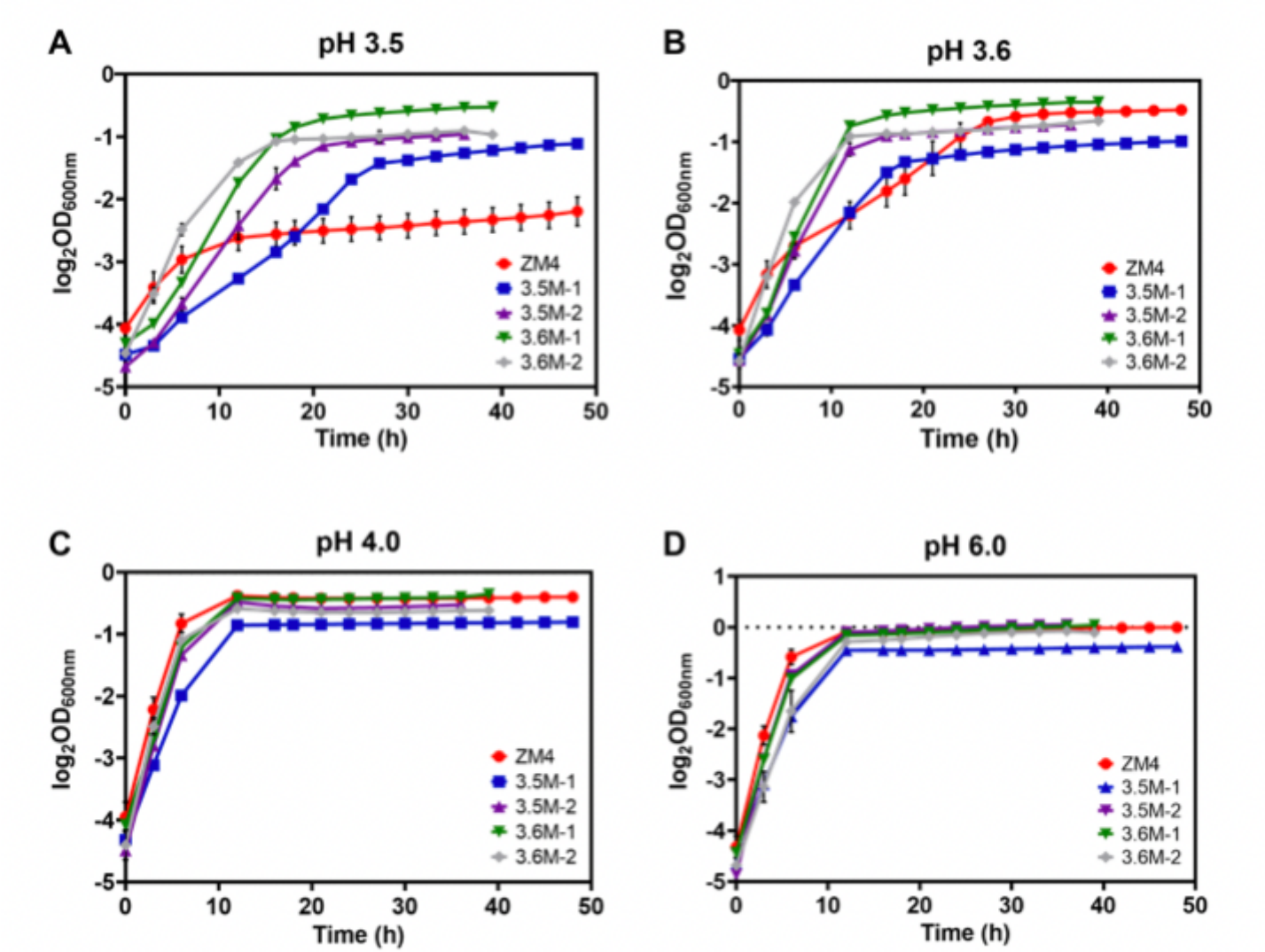

Figure 3

Cell growth of four mutants and wild-type Z. mobilis ZM4 under different pH conditions of pH 3.5 (A), pH 3.6 (B), pH 4.0 (C), and pH 6.0 (D) in RMG2. OD values at 600 nm were monitored using Bioscreen C instrument. At least two independent experiments were performed with similar results. Values are the mean of one representative experiment with three technical replicates. Error bars represent standard deviations.

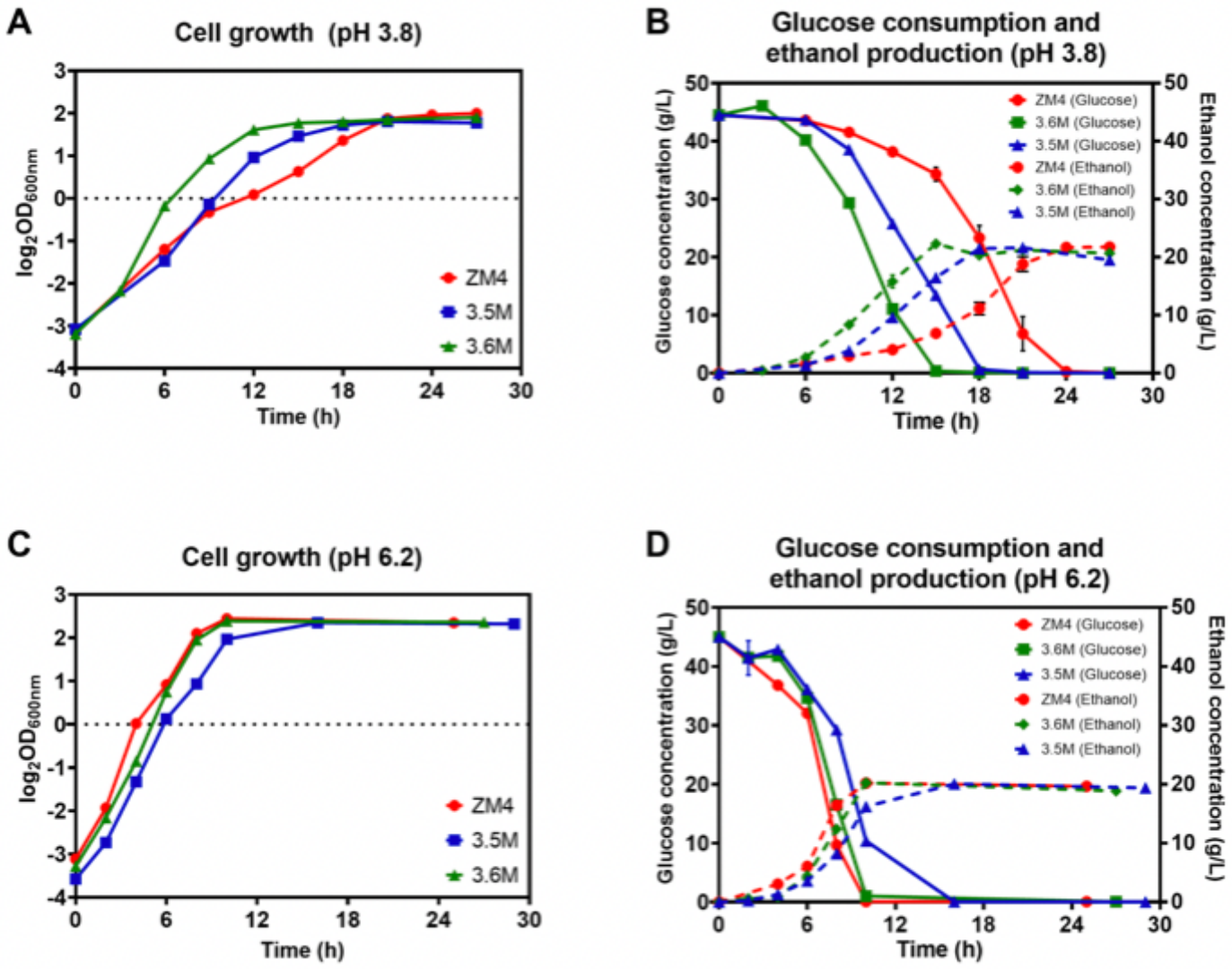

Figure 4

Cell growth, glucose consumption and ethanol production of Z. mobilis mutants 3.5M and 3.6M compared with wild-type ZM4 at pH 3.8 (A, B) and pH 6.2 (C, D). At least two independent experiments were performed with similar results. Values are the mean of one representative experiment with three technical replicates. Error bars represent standard deviations.

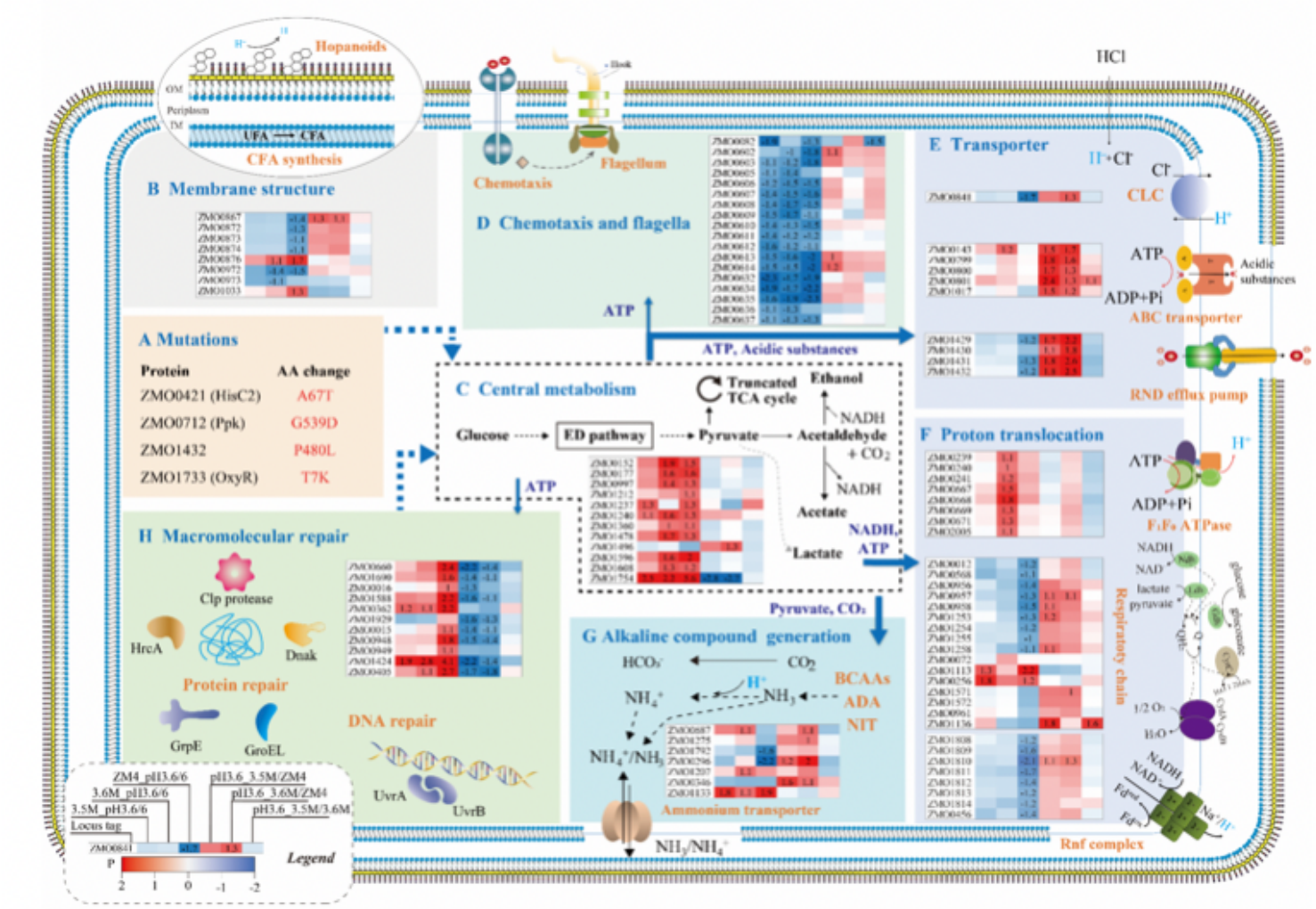

Figure 5

Potential molecular mechanism of acidic-pH tolerant mutant strains of Z. mobilis. Common mutations identified in two mutants (A); potential membrane structure changes (B); upregulation of the central metabolism producing enough ATP and reducing power (C); downregulation of chemotaxis and flagella reducing energy consumption (D); export of acidic substances by transporters (E); translocation of excess proton out of cell by F1Fo ATPase and electronic transport chain related complex (F); alkaline compound generation (G); downregulation of macromolecular repair system (H). The numbers after the gene locus in shadow represent the log2fold changes. Red indicates upregulated, blue indicates downregulated. BCAAs: branched-chain amino acids; ADA: adenosine deaminase; NIT: nitrilase.

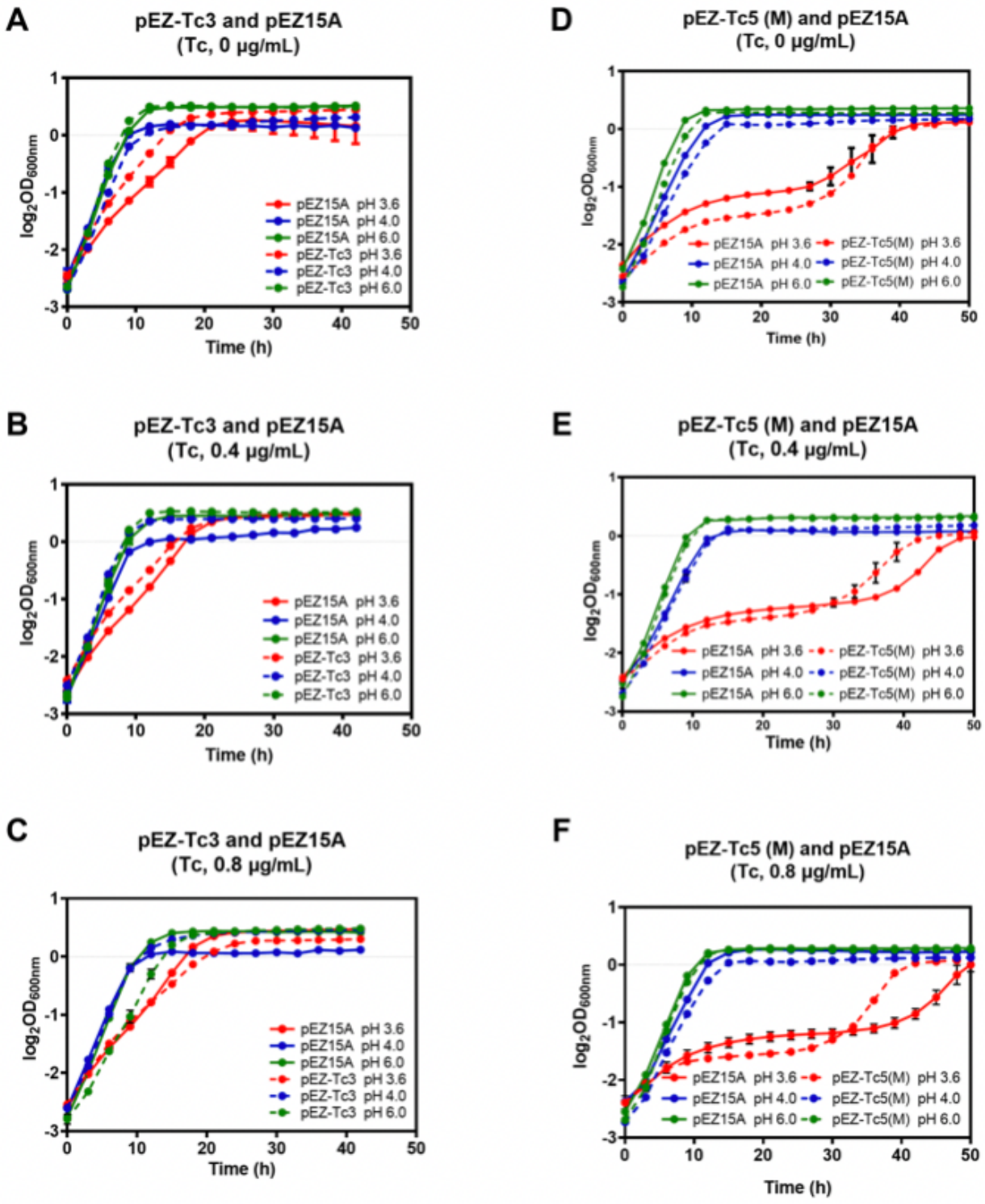

Figure 6

Cell growth of recombination and wild-type strains of Z. mobilis containing the control plasmid pEZ15A and plasmid constructs of pET-Tc3 and pET-Tc5 (M) at pH 3.6, 4.0, 6.0 with the induction of 0.8 μg/mL tetracycline (A, D), 0.4 μg/mL tetracycline (B, E), or without tetracycline induction (C, F). pEZ-Tc3, plasmid construct expressing operon ZMO0956-ZMO0958 encoding cytochrome bc1 complex; pEZ-Tc5 (M), plasmid construct expressing operon ZMO1428-ZMO1432 encoding RND efflux system with a mutation

in ZMO1432. At least two independent experiments were performed with similar results. Values are the mean of one representative experiment with three technical replicates. Error bars represent standard deviations.

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