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# Phenotypic and genomic analysis of *Zymomonas mobilis* ZM4 mutants with enhanced ethanol tolerance

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ABSTRACT

*Zymomonas mobilis* ZM4 is an ethanol-producing microbe that is constitutively tolerant to this solvent. For a better understanding of the ethanol tolerance phenomenon we obtained and characterized two ZM4 mutants (ER79ap and ER79ag) with higher ethanol tolerance than the wild-type. Mutants were evaluated in different ethanol concentrations and this analysis showed that mutant ER79ap was more tolerant and had a better performance in terms of cell viability, than the wild-type strain and ER79ag mutant. Genotyping of the mutant strains showed that both carry non-synonymous mutations in *clpP* and *spoT/relA* genes. A third non-synonymous mutation was found only in strain ER79ap, in the *clpB* gene. Considering that ER79ap has the best tolerance to added ethanol, the mutant alleles of this strain were evaluated in ZM4 and here we show that while all of them contribute to ethanol tolerance, mutation within *spo*T/*rel*A gene seems to be the most important.


## 1. Introduction

Bioethanol is considered an alternative renewable energy source, and among various ethanol-producing microbes, *Zymomonas mobilis* ZM4 (ATCC 31,821) has received special attention, mainly due to the higher ethanol yield obtained from different sugars and its inherent tolerance to ethanol [1–5]. This microorganism is capable of fermenting glucose, fructose and sucrose to produce ethanol and carbon dioxide via the Enther-Doudoroff pathway. *Z. mobilis* has many desirable industrial characteristics, such as high-specific ethanol productivity, high ethanol yield, high ethanol tolerance and a wide fermentation pH range [2,3,6–8]. Metabolic engineering can provide new characteristics and capabilities to *Z. mobilis* like expand its substrate range, remove competing pathways and enhance its tolerance to ethanol and to other lignocellulosic hydrolysate inhibitors as aliphatic acids, furan aldehydes and furfural [9–11]. However, considering the variety of factors involved in the response to high concentrations of these compounds makes it difficult to devise new metabolic engineering strategies to generate tolerant strains, and for this reason still important to find novel genes and traits related to ethanol tolerance and to other biomass-derived growth inhibitors [8–12]

Accumulation of ethanol produced during fermentation is toxic to microorganisms depending on their intrinsic tolerance. Ethanol can promote changes in membrane composition and affect membrane-related processes such as energy generation and transport. At high concentrations, ethanol inhibits cell growth and ultimately results in the death of the microorganism. Inhibition by ethanol limits product titers, affects fermentation performance and has an economic impact on the process [13,14].

To date, there are few studies related to ethanol stress in *Z. mobilis* ZM4. [15] showed that alcohol dehydrogenase II is a protein that is mainly present under ethanol stress, and [16] identified 127 genes related to ethanol stress using microarrays. These genes were mainly associated with cell wall/membrane biogenesis, metabolism and transcription. In their transcriptomic study using systems biology [17], found down-regulated genes related to translation and ribosome biogenesis, while up-regulated genes

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were related to cellular processes and metabolism. Also, it has been shown that the 5′ untranslated regions of genes ZMO0347 and ZMO1142, encoding the RNA binding protein Hfq and a thioredoxin reductase, down-regulate downstream gene expression under ethanol stress [18]. It has been also shown that some regulatory small RNAs are differentially expressed in presence of 5% of exogenous ethanol [19]. Additionally [20], showed that one of the two cell surface exopolysaccharides that *Z. mobilis* possess (PS2), may have a crucial role in ethanol tolerance.

In this study, we used a different approach to identify genes of *Z. mobilis* involved in ethanol tolerance and to describe their influence on the physiology of this organism under high ethanol concentrations. A population of *Z. mobilis* ZM4 was forced to grow under increasing ethanol concentrations to select mutants with augmented ethanol tolerance. Two mutants (ER79ap and ER79ag) with distinct morphology were isolated from this process of directed evolution. The phenotype of these mutants was evaluated, and both had a higher tolerance to added ethanol than the wild-type strain. However, ER79ap in the presence of ethanol not only has a better performance than the wild-type and ER79ag mutant in terms of ethanol tolerance, but also in cell viability, and glucose consumption, and ethanol production. The genotype of the mutants was determined by sequencing their genomes and showed that ER79ag has four mutations: *clp*P(S26 G), *rim*O(R399C), *spoT*/*relA*(R676 M), ZMO0297(V313A) and the loss of one of its plasmids. ER79ap has three non-synonymous mutations: *clp*P(S26 G), *spo*T/*rel*A(R317 G), and *clp*B(V108A). Finally, we evaluated the mutant alleles present in the genome of ER79ap by introducing them individually, on a plasmid vector, into the wild-type strain. Here we show that all mutant alleles contribute to ethanol tolerance but one of them, *spo*T/*rel*A(R317 G), seems to be more important than the others. In the absence of added ethanol, the mutants and transconjugants did not show maladaptations to ferment dextrose into ethanol using 20 or 150 g/L of glucose. Genes identified in this work can be used as new targets to generate ethanol-tolerant strains in this and other ethanologenic microorganisms.

## 2. Methods

### 2.1. Z. mobilis *strain, culture conditions and inoculum development*

*Z. mobilis* ZM4 was acquired from the American Type Culture Collection (ATCC 31821). This strain and its mutant derivatives ER79ap and ER79ag were grown in MR-MES medium, which is a modification of that reported by Bringer et al. [21]. This modified medium contained (g/100 mL $H_20$): yeast extract, 0.5; $(NH_4)_2SO_4$, 0.1; $MgSO_4{\cdot}7H_20$, 0.05 and MES (100 mM) (pH = 6). Liquid cultures were grown in 350-mL mini-fermenters [22] containing 200 mL of MR-MES without aeration at 30 °C, 100 rpm and an initial pH of 6. The inoculum was prepared from a fresh colony obtained from solid MR-MES medium (agar 15 g/L). A single colony was seeded in a test tube containing 10 mL of culture medium and was incubated overnight at 30 °C and 250 rpm. Enough culture was used to start mini-fermenter cultures at initial $OD_{600}$ values of 0.025 (Low Optical Density, LOD) or 0.25 (High Optical Density, HOD), depending on the experiment, and glucose at 20 g/L. Ethanol was added to the MR-MES liquid medium at the desired concentration, when needed. Each experiment was performed in triplicate. To determine significant differences between average values a *t*-student test was used with a p-value of 0.05. Also, an ANOVA was performed to find differences between the experiments performed at the two different inoculum conditions (p-value, 0.05). A set of cultures was performed using the ZM4, ER79ap, ER79ag, and transconjugants (see below) strains to evaluate ethanol production at high glucose concentration: 150 g/L.

### 2.2. Minimal inhibitory concentration tests

The determination of the Minimum Inhibitory Concentration (MIC) of ethanol for the ZM4 strain was performed in mini-fermenters using the conditions described above at LOD. The ethanol added to the MR-MES ranged from 60 to 105 g/L, in 5 g/L increments. The MIC of ethanol under these conditions was 75 g/L (data not shown); hence, the adaptive evolution process described below, started with 70 g/L of ethanol.

### 2.3. Adaptive evolution

The evolution experiment with strain ZM4 was performed using the sequential transfer method, during which the ethanol concentration was increased in each step. This experiment began with nineteen transfers at a concentration of 70 g/L of exogenous ethanol at HOD. The population increased its OD during the first transfers, but in the last transfers the final OD did not change. At this point, the *Zymomonas* culture was transferred to medium containing 75 g/L ethanol. This procedure was continued until no further increases in OD were observed. This step took twenty-seven transfers. The culture was then transferred to a medium with a higher ethanol concentration (77.5 g/L). In this condition, the population was still able to grow, and the culture was maintained in this way for nine additional transfers. Finally, the culture was changed to a medium containing 80 g/L of ethanol; twenty-two transfers were made under this condition. In total, 79 transfers were performed in the presence of ethanol (data not shown). During this process, the cell viability was evaluated as Colony Forming Units (CFU).

### 2.4. Isolation of mutant strains

In the last four transfers of the evolution experiment, two colony types were observed: large and small. After the last culture was made, one colony of each type was isolated and purified through three consecutive re-seedings on solid MR-MES medium. The strain forming small colonies was named ER79ap, and the strain producing large colonies (same size as its progenitor) was named ER79ag.

### 2.5. Genome sequencing and mutation validation

Genomic DNA was extracted using a Genomic DNA Purification Kit (Thermo Scientific, Waltham, Mass; USA). DNA samples were sequenced at the National University of Mexico (UNAM) at the "*Unidad Universitaria de Secuenciación Masiva y Bioinformática*". The platform used for the DNA sequencing was a Genome Analyzer GAIIx. Using this technology, 48,603,764 reads were obtained for the wild-type genome, 33,096,910 for the ER79ap genome, and 53,153,978 for the ER79ag genome. Genomes of the mutant strains and of our ZM4 clone (ZM4o) were reconstructed aligning Illumina reads against the *Zymomonas mobilis* ZM4 reference genome sequence, deposited in GenBank (NC_006526.2), using CLC Genomic Workbench 11. Reconstructed genomes were deposited in GenBank with accession numbers: CP035711, CP036466, CP036467, CP036468 (ER79ag), CP035713, CP036459, CP036460, CP036458, CP036461 (ER79ap) and CP035712, CP036463, CP036465, CP036462, CP036464 (ZM4o). Illumina reads were deposited with accession numbers: SRR8613003 (ER79ag), SRR8613005 (ER79ap), and SRR8613004 (ZM4o). Single Nucleotide Variants (SNV) were identified with MUMmer 3.0 [23]. To validate mutations identified in the genome sequencing, DNA regions containing mutated genes were amplified by PCR and sequenced using the Sanger method at Macrogen facilities (Seoul, South Korea).

### 2.6. Analytical methods

To monitor cell growth quantification of CFUs was made using MR-MES solid medium. A spectrophotometer was used to measure the optical density at 600 nm (Beckman Coulter Du 650, CA, USA). A biochemical analyzer was used to measure the concentration of residual glucose (YSI model 2700, YSI Inc., Yellow Springs, OH, USA). The quantification of ethanol in the supernatant was performed using a gas chromatograph (Agilent, 6850 series GC System, Wilmington, DE, US) as reported elsewhere [24].

### 2.7. Survival rate calculation at different ethanol concentrations

To evaluate the survival of the strains at different ethanol concentrations (85, 90, and 100 g/L), 350-mL mini-fermenters containing 200 mL of MR-MES without aeration at 30 °C, 100 rpm and an initial pH of 6, were inoculated with $3 \times 10^7$ cells/mL, to about 0.25 O.D. (HOD), and incubated for 24 h. The numbers of viable cells (s) was obtained by seeding the proper dilutions on plates of solid MR-MES medium (without ethanol) and incubating them at 30 °C. Survival was expressed as percentage of the ratio of viable cells obtained with added ethanol to viable cells obtained without the addition of ethanol at 24 h.

### 2.8. Plasmid construction, conjugations and growth conditions

The complete coding sequences of mutant alleles from strain ER79ap were amplified by PCR. PCR products flanked by PstI and XbaI sites (added in PCR primers) were inserted into plasmid vector pBBR1MCS-3 [25]. Resulting recombinant plasmids were named pBBR_*spo*T/*rel*A, pBBR_*clp*B and pBBR_*clp*P. These plasmids and the empty vector were transformed into *E. coli* S17.1 [26]. *E. coli* S17.1 transformants, each carrying one of the recombinant plasmids or the empty vector, were used as donors to introduce the recombinant plasmids into *Z. mobilis* ZM4 by conjugation.

Conjugations were made mixing 100 µl overnight (O/N) cultures of the *E. coli* S17.1 transformants with 80 µl O/N culture of ZM4, on RM-MES agar plates. Crosses were incubated at 30 °C, O/N. Dilutions of the washed cells were plated on RM-MES agar plates containing 10 µg/mL of tetracycline and 10 µg/mL of nalidixic acid for 2 days, to select transconjugants.

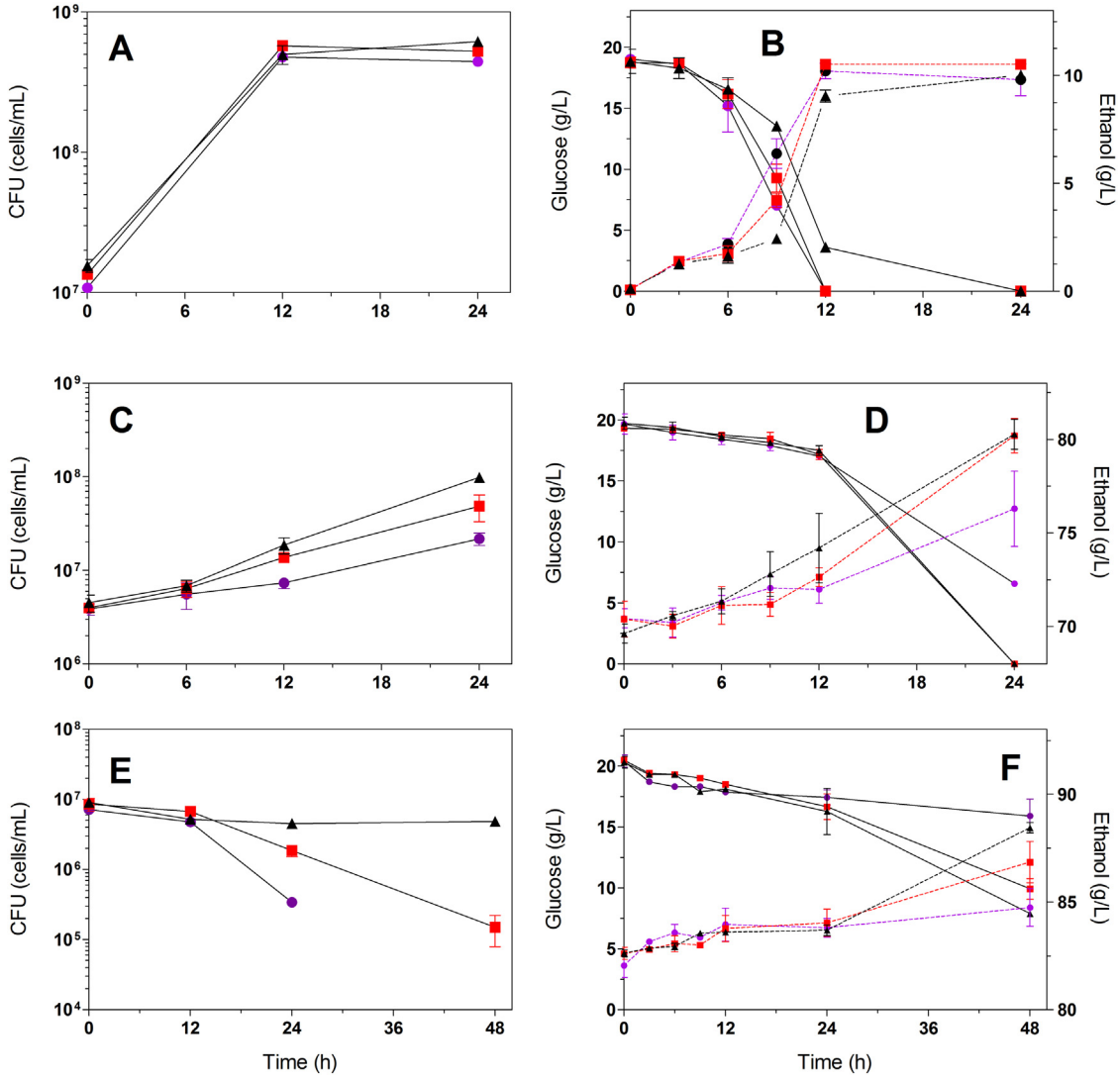


**Fig. 1.** Cultures of ER79ag, ER79ap, and ZM4 initiated at low optical density A,B) Growth, glucose consumption and ethanol production without added ethanol. C,D) Growth, glucose consumption and production of ethanol with 70 g/L of initial ethanol E,F) Growth, glucose consumption and production of ethanol with 82.5 g/L. CFU, Colony Forming Units. Triplicate cultures were used under each condition. Purple circle, ZM4; red square, ER79ag; black triangle, ER79ap.

Inocula of ZM4 transconjugants were grown for 24 h and diluted 100-fold into culture media. Two ethanol concentrations were tested in 15 x 100 mm culture tubes containing 10 mL of media. The results for each construction were expressed as a fold change of viable cells to viable cells of strain ZM4 at 24 and 48 h, on 70 and 90 g/Lof ethanol. As a control, ER79ap was also evaluated.

## 3. Results

### 3.1. Evolution experiment

*Zymomonas mobilis* ZM4 is a very efficient ethanologenic microorganism with an intrinsic high tolerance to ethanol. To obtain mutants with even higher tolerance to this solvent, this strain was cultivated in media with increasing concentrations of ethanol in consecutive steps (see methods). After 79 transfers and finishing with a culture medium containing 82.5 g/L of ethanol, the evolved population, when plated on solid media, led to the formation of two types of colonies: small and large. The strain with the capacity to form colonies with the same size as the wild-type strain was named ER79ag, and that producing small colonies was designated ER79ap. To understand the traits that increased the capacity to tolerate ethanol, the two selected mutants were phenotypically and genetically characterized.

### 3.2. Growth pattern, glucose consumption and ethanol production

First, growth (as CFU), glucose consumption and ethanol production of the wild-type and mutant strains were evaluated, using two inoculum sizes, without added ethanol. Under these conditions, we did not find statistically significant differences in CFU among the three strains. Moreover, all of them reached the same maximum CFU after 12 h of fermentation with either low or high inoculum size (Figs. 1 and 2A).

For experiments initiated with inocula at LOD, the glucose consumption of strain ER79ag was statistically similar to that of ZM4 at 9 and 12 h (Fig. 1B), while strain ER79ap consumed glucose at a slower rate, concomitantly ethanol production was also slower.

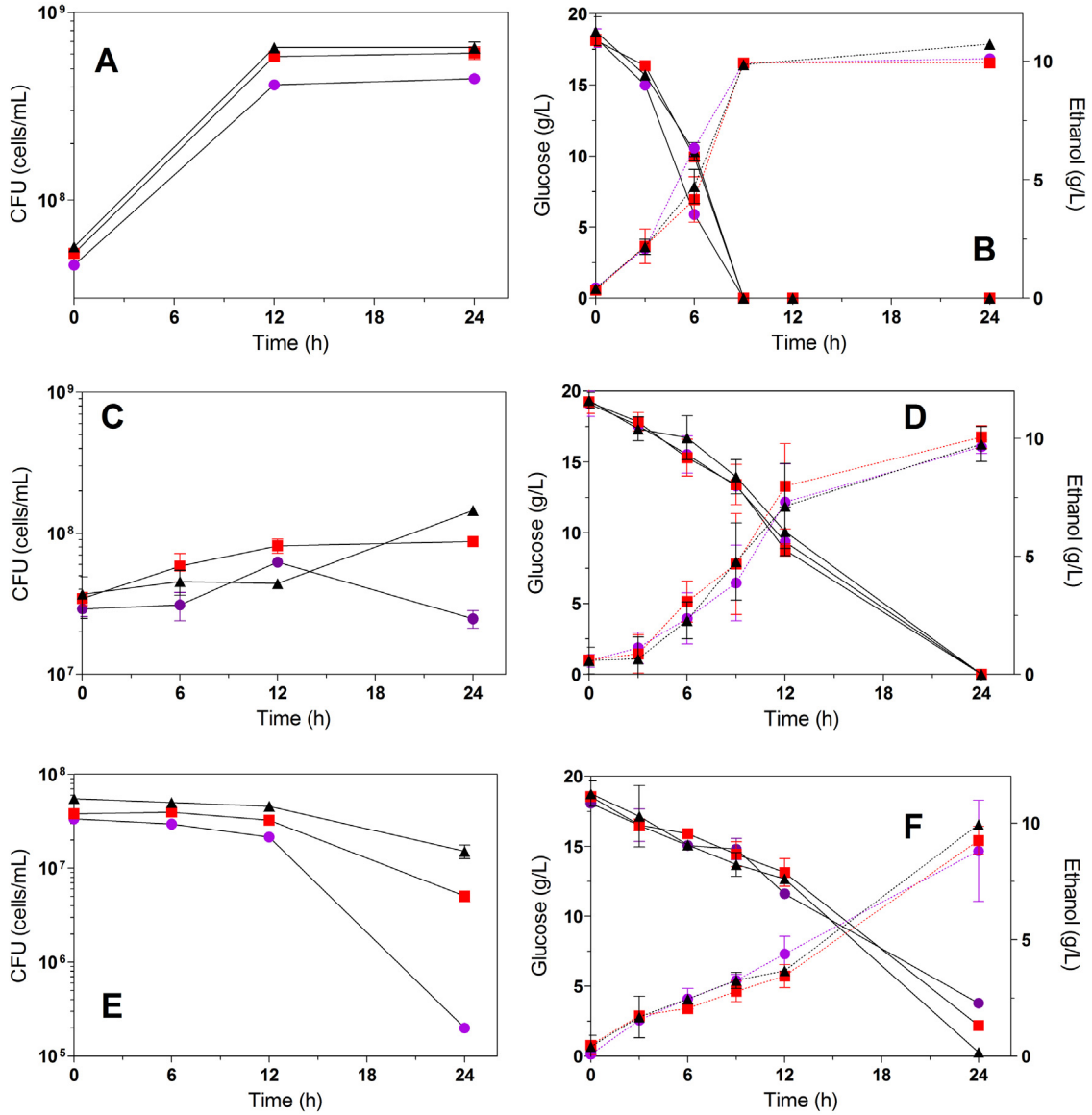


**Fig. 2.** Cultures of ER79ag, ER79ap, and ZM4 initiated at high optical density A,B) Growth, glucose consumption and ethanol production without added ethanol. C,D) Growth, glucose consumption and production of ethanol with 70 g/L of initial ethanol E-F) Growth, glucose consumption and production of ethanol with 82.5 g/L. CFU, Colony Forming Units. Triplicate cultures were used under each condition. Purple circle, ZM4; red square, ER79ag; black triangle, ER79ap.

Nevertheless, the three strains consumed all of the glucose after 24 h of fermentation (Fig. 1B) and the three reached the maximum concentration of endogenous ethanol (10 g/L) at 24 h without statistically significant differences. One of the differences using the high inoculum condition, as expected, is a faster glucose consumption rate than with the low inoculum for the three strains. Beginning the experiments at HOD, rates of glucose consumption and ethanol production were slightly slower in both mutants, but the same ethanol titers were reached after 9 h of fermentation, and glucose was also completely consumed. (Fig. 2B).

### 3.3. Effect of exogenous ethanol on growth, glucose consumption and ethanol production

At conditions of LOD and 70 g/L of added ethanol, after 24 h of cultivation, strains ER79ap and ER79ag had 3.9 and 1.5-fold statistically significant higher CFU values than the wild-type strain (Fig. 1C) and the concentration of exogenous ethanol also caused a decrease in the utilization of glucose in the parental strain, which left 6.58 g/L of residual glucose when evaluated at 24 h. In contrast, the mutants completely consumed the glucose; therefore, ethanol production in the parental strain was lower than those obtained with the mutants (Fig. 1D). On the other hand, at conditions of HOD the CFU in ER79ap and ER79ag were also higher (five-fold and three-fold respectively) than the wild-type strain (Fig. 2C). In other words, mutants have a better performance in the presence of 70 g/L of added ethanol than the wild-type strain. Strain ER79ap is the best under these conditions in terms of CFU, rates of glucose consumption and ethanol production, especially when the experiments are initiated with HOD.

Differences between the wild-type and mutant strains were evident when strains were challenged with 82.5 g/L, especially after 24 h of cultivation. Mutants had statistically significant higher rates of survival, consumed more glucose, and produced ethanol at statistically significant higher rates than the wild-type strain. (Fig. 1E,F). However, none of these strains were capable of consuming all of the glucose in the medium after 48 h of fermentation. Under these conditions, mutant ER79ap had a better performance than mutant ER79ag. For example, CFUs of ER79ap were one order of magnitude higher than those of ER79ag after 24 h of cultivation.

When experiments were done with inocula at HOD and 82.5 g/L of added ethanol, the contrast between wild-type and mutant strain was even clearer: mutant strains have a higher survival rate, measured after 24 h of cultivation, than the wild-type (Fig. 2E), and the CFU of mutant ER79ap was three times higher than the other mutant. At 24 h, ER79ap was the only strain capable of consuming all of the glucose, while the wild-type strain consumed the least (Fig. 2F).

### 3.4. Survival at high ethanol concentrations

*Z mobilis* is able to produce high concentrations of endogenous ethanol (up to 85 g/L (11% v/v) for continuous culture and up to 127 g/L (16% v/v) in batch culture [3,4]. However, there are few studies about the tolerance under exogenous ethanol conditions [16,17,19,18]. To evaluate the intrinsic capacity of ZM4 wild-type strain and its mutant derivatives, the strains were challenged with 85, 90 and 100 g/L of *exogenous* ethanol, initiating the experiments with inocula at HOD. Viability of the strains were recorder after 24 h of cultivation. As seen in Fig. 3, the mutant strains, especially ER79ap, have a much higher survival percentage than ZM4, confirming that the ER79ap mutant is more tolerant than its parental strain at ethanol concentrations above 85 g/L.

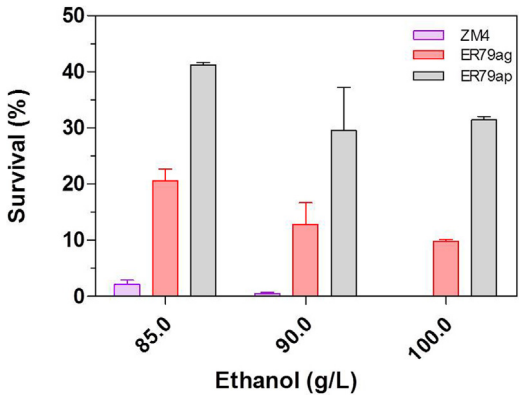


**Fig. 3.** Survival at different ethanol concentrations of ZM4 and mutant derivatives. Calculations were made at 24 h of incubation at the indicated ethanol concentrations. Triplicate cultures were used under each condition.

### 3.5. Genome sequence analysis

The genome sequences of strains ER79ap and ER79ag were obtained to identify the mutations generated through the evolutionary process. Our ZM4 clone (ZM4o) was also re-sequenced to rule out possible mutations from manipulations and storage in the laboratory. In fact, we found twenty differences in the chromosome of the re-sequenced strain that are not present in the ZM4 reference genome (NC_006526.2). Notwithstanding, mutants ER79ag and ER79ap also carry these differences and for this reason they were not considered in our analyzes (Supplementary Table_1). Also, we reconstructed plasmid sequences of our ZM4 clone and the mutant strains, using as references sequences NZ_CP023683.1 to NZ_CP023686.1 [27]. We found dozens of putative changes, but in tight clusters suggesting regions of low sequence quality rather than real SNVs. In fact, the sequence coverage of plasmids was low, contrasted to the coverage obtained for the chromosome. Changes found in plasmids laid in intergenic regions or in genes encoding hypothetical or poor characterized proteins and were not taken into consideration for posterior analyzes. Chromosome and plasmid differences are listed in Supplementary_Table_2.

As shown in Table 1, four non-synonymous substitutions were found in the ER79ag chromosome that were not present in our ZM4 strain: one of them (R676 M) was located within the ACT regulatory region of *spoT/relA*, a gene encoding a protein involved in the synthesis and degradation of the (p)ppGpp alarmone. Another mutation (S26 G) lies within in the gene encoding ClpP, a protease that, in association to the unfoldases ClpA and ClpX, degrades a wide variety of substrates and plays important roles in stress tolerance in many microorganisms [28] A third mutation (R399C) was located within in *rimO*, a gene encoding a methylthiotransferase of the 30S ribosomal protein S12 [29]. The last non-synonymous substitution (V313A) was located within a gene encoding a member of the HlyC/CorC family transporter protein (ZMO_RS01280). ER79ag also lost one of its plasmids (pZZM402).

In contrast, ER79ap chromosome possessed three non-synonymous substitutions: the first mutation (S26 G) was located in the gene encoding ClpP and was exactly the same mutation and position as that present in ER79ag. The first substitution occurred in the gene encoding the ClpB chaperone (V108A), a protein involved in suppression and reversal of protein aggregation in conjunction with other heat-shock proteins. The last substitution also fell within the *spoT/relA* gene (R317 G), but in the synthesis/degradation region.

**Table 1**
Mutations found in ER79ag and ER79ap. Comparison was made with the sequence of ZM4 clone sequenced in this work (ZM4*). Positions listed here are the same that those in ZM4 reference sequence (NC_006526.2). **P_ZM4*** position in ZM4*; **P_ER79ag**, position in mutant ER79ag; **P_ER79ap**, position in mutant ER79ap; **P_CHANGE**, amino acid residues sequence change involved in the mutant protein.

| P_ZM4* | SUBST. | P_ER79ag | LOCUS_TAG | PROTEIN_ID | PRODUCT | P_CHANGE | INTERGENIC_REGION |
|---|---|---|---|---|---|---|---|
| 76942 | C A | 76942 | ZMO_RS00360 | WP_012817506.1 | Bifunctional (p)ppGpp synthetase/guanosine-3',5'-bis (diphosphate) 3'-pyrophosphohydrolase, **SpoT/RelA** | R676M | __ |
| 299718 | T C | 299718 | ZMO_RS01280 | WP_011240232.1 | **HlyC/CorC** family transporter | V313A | __ |
| 810750 | G A | 810750 | ZMO_RS03600 | WP_011240680.1 | 30S ribosomal protein S12 methylthiotransferase **RimO** | R399C | __ |
| 965913 | A G | 965913 | ZMO_RS03600 | WP_011240805.1 | ATP-dependent **Clp** protease proteolytic subunit | S26G | __ |
| 1612573 | A G | 1612573 | __ | __ | __ | __ | ZMO_RS07065.. ZMO_RS07070 |
| **P_ZM4*** | **SUBST.** | **P_ER79ap** | **LOCUS_TAG** | **PROTEIN_ID** | **PRODUCT** | **P_CHANGE** | **INTERGENIC_REGION** |
| 78020 | G C | 78020 | ZMO_RS00360 | WP_012817506.1 | Bifunctional (p)ppGpp synthetase/guanosine-3',5'-bis (diphosphate) 3'-pyrophosphohydrolase, **SpoT/RelA** | R317G | __ |
| 965913 | A G | 965913 | ZMO_RS04250 | WP_011240805.1 | ATP-dependent **Clp** protease proteolytic subunit | S26G | __ |
| 971444 | C T | 971444 | ZMO_RS09165 | __ | IS5/IS1182 family transposase/ pseudogene | __ | __ |
| 1438735 | T C | 1438735 | ZMO_RS06375 | WP_011241206.1 | ATP-dependent chaperone **ClpB** | V108A | __ |
| 1612573 | A G | 1612573 | __ | __ | __ | __ | ZMO_RS07065.. ZMO_RS07070 |

### 3.6. Effect of the mutant alleles on ethanol tolerance

Taking in consideration that strain ER79ap has the best performance when confronting exogenous ethanol, we made an evaluation of the contribution of each one of the mutant alleles to ethanol tolerance. To do this, *clpP*, *clpB* and *spo*T/*rel*A mutant alleles of ER79ap were amplified by PCR, cloned in a replicative vector (pBBR1-MCS3) and introduced to the wild-type strain by conjugation. As a control, the empty vector was also introduced into ZM4. Transconjugant strains were evaluated with inocula at LOD and in culture media with 70 and 90 g/L of exogenous ethanol (Figs. 4 and 5).

At 70 g/L of added ethanol, ER79ap was the strain with the best growth rate. In comparison with the wild-type strain, this strain produced 2.19-fold at 24 h and 4.17-fold CFU/mL at 48 h. ZM4 derivatives carrying the mutant genes on plasmids also had a better performance than the wild-type strain: ZM4 pBBR-*spo*T/*rel*A strain showed a 1.38-fold increase in CFU/mL over the control at 24 h and 2.2-fold at 48 h, while ZM4 pBBR-*clp*B had an increase of 1.38- fold at 24 h and 2.03-fold at 48 h; and finally the ZM4 pBBR-*clp*P strain had a 1.24-fold increase in CFU at 24 h and 1.93-fold at 48 h. (Fig. 4). These results show that mutant alleles were dominant against their cognate wild-type alleles and that the three genes made a contribution to ethanol tolerance.

At 90 g/L of ethanol the mutant strain has the higher survival rate than the control strain, but ZM4 pBBR-*spo*T/*rel*A strain had 10-fold higher values of CFU/mL over the control strain at 24 h. At this time ZM4 pBBR-*spo*T/*rel*A has an even higher survival rate that the mutant strain ER79ap. Strains ZM4 pBBR-*clp*B and the ZM4 pBBR-*clp*P do not produce an increase in the number of CFU over the control at any time (Fig. 5). These results suggest that mutant allele *spo*T/*rel*A (R317 G), has a higher contribution in ethanol tolerance

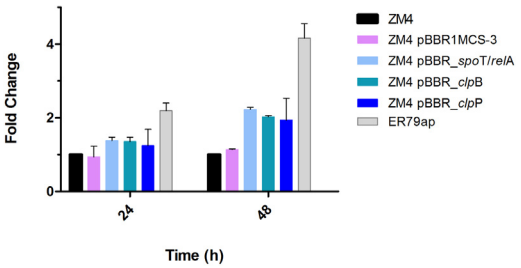


**Fig. 4.** Fold change of ER79ap, and complementary strains in presence of 70 g/L of ethanol. Triplicate cultures were used under each condition.

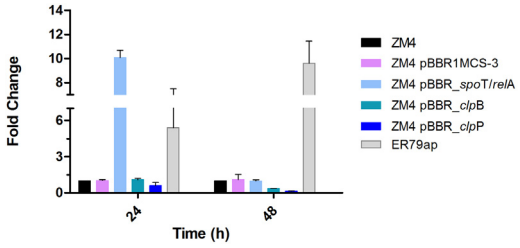


**Fig. 5.** Fold change of ER79ap, and complementary strains in presence of 90 g/L of ethanol. Triplicate cultures were used under each condition.

than the other mutant alleles studied here. But, to understand why the difference is so high at 24 h, but not a 48 h, will require more experimentation.

### 3.7. Ethanol production at high glucose concentration

Growth (as CFU), glucose consumption and ethanol production, using the wild-type, mutants and transconjugant strains, were evaluated at 150 g/L of glucose, using low inoculum size (LOD = 0.025) (Fig. 6). During the first 12 h of fermentation elapsed time, the ER79ap strain and the transconjugant expressing *spo*T/*rel*A developed a lower amount of CFU (Fig. 6A); but, similarly to the experiment using low glucose concentration, at the time of glucose depletion (Fig. 6B) most of the strains reached their maximum growth at 24 h of cultivation. No statistically significant differences in CFU between the six strains was found at 24 h. Likewise, the glucose consumption of strain ER79ag and transconjugants was like that of ZM4 (Fig. 6B), while strain ER79ap consumed glucose at a little bit slower rate, concomitantly ethanol production was also a little bit slower. Hence, except for ER79ap, the strains consumed all of the glucose at 24 h of fermentation reaching the maximum concentration of produced ethanol (in average 72.36 g/L +/- 0.72; i.e. a yield equivalent to 95% of the theoretical) at 24 h. Furthermore, to evaluate tolerance to endogenous ethanol in the absence of glucose, the cultures were continued after maximum ethanol production and the CFU were measured after 24 h of glucose depletion (Fig. 6A). It can be observed that after 24 h of exposure to an average value of 72 g/L of ethanol the strain ER79ap showed the highest number of CFU, followed by the transconjugant carrying the *spo*T/*rel*A allele, and the wild type strain; while the strain carrying the *clp*P allele had the lowest number of CFU.

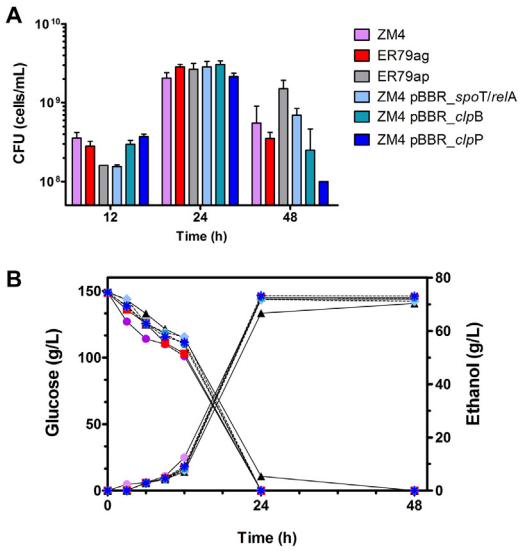


**Fig. 6.** Cultures of wild-type, mutants (ER79ag, ER79ap) and transconjugants (ZM4 pBBR_*spo*T/*rel*A, ZM4 pBBR_*clp*B, ZM4 pBBR_ *clp*P) strains initiated at low optical density (0.025 OD) and high glucose concentration (150 g/L). A) Growth of strains as colony forming Units (CFU). B) Glucose consumption and ethanol production. Triplicate cultures were used under each condition. Purple circle, ZM4; red square, ER79ag; black triangle, ER79ap; diamond, ZM4 pBBR_*spo*T/*rel*A; hexagon, ZM4 pBBR_*clp*B; star, ZM4 pBBR_*clp*P.

## 4. Discussion

Ethanol is a fermentation product that is toxic to cells at some point, even for organisms that produce it. For this reason, high ethanol tolerance is an advantageous property in organisms used for industrial production. The mechanisms of ethanol tolerance are well understood in *Saccharomyces cerevisiae* [30,31]. However, our knowledge as to how bacteria contend with ethanol tolerance is limited to a few studies principally performed in *Escherichia coli* [32–35] and *Zymomonas mobilis* [15–1936].

*Z. mobilis* ZM4 is an ethanologenic microorganism with an intrinsic higher tolerance to ethanol. It is known that ethanol accumulation reduces the capacity of this bacterium to convert glucose to ethanol [34,37]. Ethanol accumulation in *Z. mobilis* also induces changes in some protein components of the cell membrane and in proteins related to the heat shock response [38]. Recently, He and coworkers [16] performed a transcriptional analysis of *Z. mobilis* ZM4 under ethanol stress. They identified 127 genes that were differentially expressed under ethanol stress that belonged to a wide variety of functional classes, including genes that participate in carbohydrate metabolism, cell wall and membrane biogenesis, terpenoid biosynthesis, DNA replication, recombination and repair, transport, and transcriptional regulation. Similarly, Yang et al. [17] showed that ZM4 ethanol response involve a wide variety of genes that belong to different functional categories: most ethanol-upregulated genes were clearly involved on cellular processes and metabolism and in contrast, down-regulated genes were related to translation and ribosome biogenesis. Also, it has been recently shown that some regulatory small RNAs are differentially expressed in presence exogenous ethanol [19], and that the 5′ untranslated regions of some genes have a regulatory role under ethanol stress [18].

In this work, to achieve a better understanding of ethanol tolerance in *Z. mobilis* ZM4, we obtained two mutant derivatives with higher tolerance to ethanol through a process of directed evolution. We showed that these mutants had distinct colony morphologies: ER79ap formed small colonies and ER79ag produced colonies of the same size as the parental strain. The colony size of ER79ap is reminiscent of the phenomenon of small colony variants (SCVs) described for some bacterial pathogens that, in addition to their reduced colony size, are capable of resisting various types of stress [39].

Both strains were capable of growth beginning with low inoculum (0.025 OD) in the presence of 82.5 g/L of ethanol and survived for longer periods (at 24 h) than the wild-type strain in the presence of 90 g/L of ethanol. However, ER79ap has a much better performance confronting added ethanol than ER79ag. Furthermore, efficient fermentation of glucose to ethanol at low (20 g/L) or at high glucose concentration (150 g/L) was not affected in the evolved strains or in transconjugants strains, expressing three different alleles found in the ER79ap. The set of mutations developed in ER79ap delayed a little bit glucose consumption and ethanol production, in comparison to the progenitor strain. However, the level of ethanol obtained was the same from the same amount of glucose.

Therefore, the mutants and transconjugants did not show maladaptations to glucose conversion into ethanol. Notwithstanding, ethanol tolerance did not crosslinked with other types of stresses when tested in solid media: ER79ap was more sensible than the wild-type strain when confronted with concentrations of NaCl below the inhibitory threshold (2.5 and 5 g/L), with high concentrations of glucose (250 g/L) or to moderate concentrations of antibiotics, such as streptomycin, tetracycline and nalidixic acid (data not shown). As described above, when cultivated in liquid media to test ethanol production, cell viability was statistically significantly higher for ER79ap than ZM4 once glucose was depleted and ethanol concentration was close or above 70 g/L. Additionally, ER79ap flocculated more easily than the wild type and ER79ag strains (data not shown). Flocculation is also characteristic of industrial interests because it facilitates biomass recovery [40,41].

During the evolution process, ER79ag lost one plasmid and acquired mutations in the *spoT/relA, clpP, rimO* and the gene encoding a HlyC/CorC family transporter protein (ZMO_RS01280). In comparison, ER79ap gained mutations in *clpB* and, similar to the other mutant strain, also acquired mutations in the *relA/spoT* and *clpP* genes. Both mutants had the same mutation in the same position in their *clpP* gene, indicating that these strains diverged from a common ancestor during the evolution process. Further, we consider it relevant that the two strains have mutations in the same gene but in different places. In ER79ag, the mutation is located at the *spoT/relA* ACT regulatory module, and the mutation in ER79ap resides within the synthesis module. This difference in the position of the mutation might be contributing to the increase in tolerance in ER79ap strain. These observations suggest that mutations in *clpP*, but particularly in the *spo*T/*rel*A gene, have a central role in the enhanced ethanol tolerance that the mutants exhibit [42].

The role of the SpoT/RelA protein is to synthesize and degrade the (p)ppGpp alarmone, a metabolite that has a crucial role in the stringent response and other types of stress. Typically, an increase in (p)ppGpp levels induces a radical change in the transcription profile, characterized by repression of genes involved in rapid growth, such as rRNA, tRNA genes and ribosome protein genes; cell division; cell motility; metabolite transport; translation initiation and elongation; and increased expression of genes involved in amino acid synthesis, nutrient uptake, carbohydrate synthesis and other functions [43–46].

ClpB is an AAA + ATPase member of the Clp/Hsp100 family that, in conjunction with other chaperones, inhibits and reverses protein aggregation [47]. This protein has an important role in cellular tolerance to different types of stress, including ethanol in

*Brucella suis* [48], and likely also has a role in ethanol tolerance in *Z. mobilis*. ClpP is a protease that, in association to the unfoldases ClpA and ClpX, degrades a wide variety of substrates and plays important roles in stress tolerance in many microorganisms [28,49,50].

To evaluate the role of the mutant alleles on enhanced ethanol tolerance of ER79ap, we constructed three derivatives of ZM4, each one carrying *in trans* one of the mutant alleles present in the mutant strain. The growth and/or survival rate of these derivatives and of the parental strain were evaluated in presence of 70 and 90 g/L of exogenous ethanol. Overall, our observations indicate that ER79ap had better performance, in terms of CFU, when confronting added ethanol in comparison to the other strains. Derivatives carrying the mutant alleles *in trans*, grew better and had a higher survival rate at 70 g/L than ZM4, indicating not only that the three alleles are dominant over the wild-type alleles, but that all of them contributed to ethanol tolerance. Nevertheless, at 90 g/L of ethanol strain ZM4 pBBR-*spoT/relA* at 24 h post-inoculation had 10 times higher survival rate than the control strain, but at 48 h this advantage disappeared, suggesting that this allele, in some conditions, has a crucial contribution to ethanol tolerance. However, strain ER79ap showed higher survival rates than the control strain at 24 and 48 h, suggesting that the combination of mutations has a synergistic effect over ethanol tolerance. Undoubtedly, more experiments will be needed to elucidate the molecular mechanisms in which these mutant proteins are involved in ethanol tolerance. However, we can hypothesize that mutations in *clp*P and *clp*B improve the activity to contend with the aggregated or misfolded proteins generated under ethanol stress and considering that (p)ppGpp has been implicated in not only the stringent response but also other types of stress, we suggest that an increase in the basal synthesis of (p)ppGpp is involved in the tolerance properties of ER79ap. We propose that the *spoT/relA* mutant alleles obtained here are able to synthesize more easily (p)ppGpp or degrade this compound more slowly, promoting that mutants respond more rapidly to added ethanol. For biotechnology purposes, our work indicates that *spoT/relA*, *clpB*, and *clpP* can be used as new targets, individually or in combination, to engineer strains with increased ethanol tolerance.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

OEC-R conceived the study, performed the experiments, made the data analysis and designed the figures and drafted manuscript. JLA made the analysis of the genome sequences. OEC-R, AM, RMG-R, and MAC designed the experiments, performed the data analysis, drafted and edited the manuscript. All authors read and approved the final manuscript.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2019.e00328.

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