Periplasmic SacB as a robust counter-selection tool for genome engineering in the polyploid bacterium *Zymomonas mobilis*

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Abstract

The alpha-proteobacterium *Zymomonas mobilis* exhibits exceptional ethanologenic physiology, which makes it a traditional alcoholic beverage producer and a promising chassis for biofuel production. Although genetic tools for this organism have expanded in recent years, a fundamental aspect of its chromosome organization remains to be understood. In particular, *Z. mobilis* has been suggested to exhibit polyploidy, but this feature is not fully confirmed because of discrepancies among studies reporting the copy number of chromosomes. Here, we tagged the chromosome-partitioning protein ParB with a fluorescent marker to visualise its cellular localisation and estimate chromosome copy number in individual cells.

Imaging showed that *Z. mobilis* exhibits several distinctive ParB foci throughout the cytoplasm and an accumulated focus at the pole, demonstrating that a single *Z. mobilis* cell contains at >5 copies of the chromosome at the *oriC* regions. After verifying its polyploidy, we sought to establish an efficient counter-selection system, which is crucial for engineering multiple copies of the chromosome. We assessed the efficacy of levan-sucrase (SacB) toxicity in *Z. mobilis*. We found that, despite *Z. mobilis* secreting a native extra-cellular sucrase SacB, heterologous periplasmically-localised *Bacillus subtilis* SacB rendered *Z. mobilis* cells sensitive to sucrose. We successfully used this effect for counter-selection when deleting and inserting targeted DNA sequences into the *Z. mobilis* genome. Together, this work provides important insights and tools for advancing *Z. mobilis* genetics and its biotechnological applications.

Importance

Zymomonas mobilis is a promising industrial bacterium with capacity to convert sugars into ethanol at nearly maximum theoretical yield. With its expanding use in industrial applications, it is crucial to clarify if individual *Z. mobilis* cells carry multiple copies of chromosome as this has important implications for genome engineering. Two previous studies have used quantitative PCR to address this question, but their reported chromosome copy numbers varied widely from 20 to 100. Here, we used a cell biological approach to estimate the copy number and confirmed that single *Z. mobilis* cell possesses multiple copies. In addition, we show that a SacB-based counter-selection works in *Z. mobilis*, enabling efficient and complete mutation of all chromosome copies.

50 **Introduction**

51 The alpha-proteobacterium *Zymomonas mobilis* is a natural ethanologen with an
52 exceptional metabolism that can convert sugars into ethanol nearly at the maximal
53 theoretical yield. Its carbon metabolism, the Entner–Doudoroff pathway coupled to
54 pyruvate decarboxylase and alcohol dehydrogenase, is both specific and very rapid
55 (1, 2). These features make *Z. mobilis* one of the most promising chassis organisms
56 for bioethanol production. Beyond ethanol, *Z. mobilis* has recently been engineered
57 to produce various high-value chemicals (3-9).

58 In efforts to develop *Z. mobilis* for industrial applications, the available genetic tools
59 have expanded considerably in recent years. However, its basic chromosome
60 organisation at the cellular level remains poorly characterised (10). Several studies
61 have suggested that *Z. mobilis* carries multiple copies of its chromosome and is
62 therefore a polyploid species. The first indication of polyploidy came from the
63 transposon mutagenesis study, which revealed insertions in genes considered
64 essential (11). Two other studies using quantitative PCR showed that *Z. mobilis*
65 possesses multiple copies of the chromosome (12, 13). However, the estimated
66 average number of chromosomes per cell varied considerably between reports. Our
67 previous study showed that strains Z6 and Z4 contain approximately 15–20 copies at
68 the replication origin during active growth (12), whereas another study using the
69 same qPCR based method reported that strain Z4 may carry 60 - 100 copies (13).
70 This significant discrepancy required clarification as the presence of multiple
71 chromosome copies can complicate genetic manipulation. When the applied
72 selection is not tight enough, mixed genotypes are generated, with mutant genomes
73 co-existing with wild-type copies in individual cells. This phenomenon has been
74 reported in previous studies attempting to engineer the *Z. mobilis* genome (14-16).

75 To address this, we employed a complementary approach to estimate the
76 chromosome copy number: visualising a defined locus using a fluorescent marker
77 and directly counting the signals per single cells (17). This method also offers
78 insights into the spatial organisation of chromosomes (18). Using this approach, we
79 gained further insights in its chromosomal organisation and polyploidy.

80 Furthermore, having confirmed the polyploidy of *Z. mobilis*, we recognised the need
81 for a robust counter-selection method to enable efficient introduction of desired
82 genetic changes into all chromosomal copies (19). We assessed and successfully
83 applied a SacB-based counter-selection in engineering the *Z. mobilis* genome.

84

85 **Material and methods**

86 **Bacterial strains, plasmids, and growth conditions**

87 Bacterial strains and plasmids used in this study are listed in Table S1. *Z.*
88 *mobilis* was grown in RM complex medium containing glucose (20 g/L), yeast extract
89 (5 g/L), NH_4SO_4 (1 g/L), KH_2PO_4 (1 g/L), and $MgSO_4$ (0.5 g/L). The complex medium
90 was briefly sparged with nitrogen gas to make it micro-aerobic. 12 ml of anaerobic *Z.*
91 *mobilis* culture were grown at 30°C in a capped 15 ml Falcon tube without
92 shaking. For plotting growth profiles, the optical density was measured in a 96-well
93 plate by SPECTROstar Nano (BMG labtech).

94 **Construction of *Z. mobilis* strains**

95 For all *Z. mobilis* strains, we used a counter-selection method based on the
96 previously published method (20). Crucially, we used an integrating vector (suicide
97 vector) that carried *Bacillus subtilis sacB* for negative selection (pPK15534 +
98 $SacB^{BS}$). To construct the vector, we amplified the *sacB* gene with its native promoter

region from *B. subtilis* genomic DNA using Q5 DNA polymerase (New England Biolabs). The product was column purified (QIAquick PCR Purification Kit, Qiagen) and introduced into the suicide plasmid pPK15534 by Gibson assembly (New England Biolabs) (20, 21).

To construct plasmids for gene deletion, the upstream and downstream regions (500 – 1000 bp in size) of the gene of interest, in addition to homologous regions required for Gibson assembly were amplified using Q5 DNA polymerase. For insertion of a tag, the upstream and downstream regions of the insertion sites and the tag were amplified. The obtained DNA fragment was inserted into the linearised plasmid pPK15534 + $sacB^{BS}$ by Gibson assembly. The sequences of all constructed plasmids were verified by Sanger sequencing (Eurofins Genomics).

Transfer of the suicide vector was performed via conjugation from *E. coli* as described (20). Briefly, the actively growing recipient *Z. mobilis* cells were mixed with *E. coli* WM6026 strain carrying the suicide plasmid, and then plated on RM agar medium with 0.1 mM meso-diaminopimelate (DAP) and 2% glucose overnight at 30 °C. The next day, the grown cells were scraped and resuspended in liquid RM + 2% glucose, followed by an incubation at 30°C for 2 h. Cultures were then diluted (typically 10x and 100x, depending on the density) into phosphate-buffered saline (PBS) and 100 µL of each dilution was spread on RM + 2% glucose agar medium with 90 µg/mL chloramphenicol to select for the cells that received and integrated the plasmids.

The colonies that grew on the selection plates were then checked for the presence of plasmid in their chromosomal DNA by colony PCR. Verified colonies were then grown in liquid RM + 2% glucose without selection overnight (excision of the

123 plasmids). Next day, the cells were diluted in PBS (10, 100 and 1000x), and 100 µL
124 from each diluted culture were spread on RM agar supplemented with sucrose
125 (initially using 3%, 4% or 5%) and the plates were incubated for two days. We found
126 that 3% sucrose provided tight selection and used this concentration for generating
127 most of the strains in Table 1, but 5% sucrose was also effective. After the colonies
128 appeared, single colonies were streaked on RM with 2% glucose, then colony PCR
129 was performed to identify colonies with the desired genotype. The genomic DNA of
130 several generated strains were extracted using a genomic DNA kit (Sigma),
131 sequenced by whole genome sequencing (MicrobesNG) and analysed by CLC
132 Genomics Workbench (Qiagen). Oligonucleotides used in this study are listed in
133 Table S2.

134 **Microscopy analysis**

135 A culture of growing *Z. mobilis* (0.4 µl) was spotted onto a 1% agarose-pad made of
136 RM growth medium and covered by a small cover-glass. Phase contrast and
137 fluorescence images of *Z. mobilis* cells were taken using a Nikon Eclipse Ti
138 microscope (Nikon) equipped with a Plan Apochromat 100x objective with an NA of
139 1.40 and Ph3 Phase plate (Nikon) and a Prime sCMOS camera (Teledyne
140 Photometrics). ParB-sfGFP and FM 4-64 were imaged using 49002 and 49008 filter
141 cubes respectively (Chroma). Images were acquired using Nikon NIS elements AR
142 software. Image analysis was performed using MicrobeJ (22). For chromosome foci
143 counting, we used Maxima function in MicrobeJ using tolerance value as 850. For
144 staining membrane, the cells were incubated with FM4-64 (2 µg/mL), for 5 minutes
145 prior to imaging.

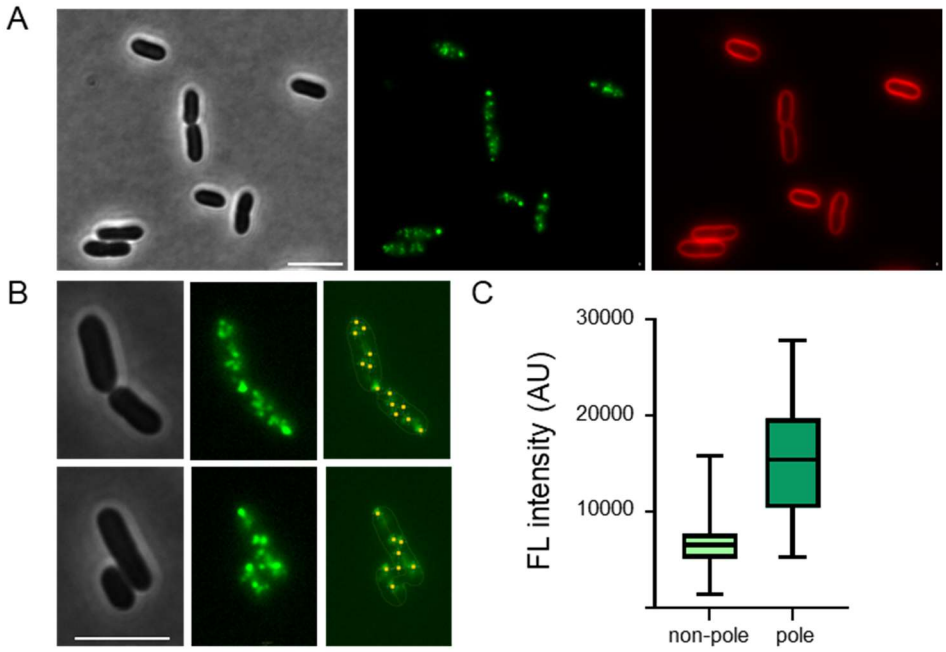
146

147 **Results**

148 **ParB-sfGFP forms multiple foci in single *Z. mobilis* cells**

149 To visualise the *oriC* site of chromosome in *Z. mobilis* cells, we expressed
150 chromosome partitioning protein ParB fused to the fluorescent protein sfGFP (super-
151 folder variant of GFP) (23). ParB has been shown to play a central role in
152 segregating of replicating chromosomes in several bacteria, assembling at *parS* DNA
153 sites located near the origins of replication (*oriC*) (24). Bioinformatic analysis
154 predicted four *parS* sites near the *oriC* in the *Z. mobilis* chromosome (25). Thus,
155 ParB sub-cellular localisation should serve as a marker for counting chromosomal
156 origins in a single *Z. mobilis* cell. (17) We generated a *Z. mobilis* strain in which
157 *parB-sfGFP* was introduced at the original locus (*Z6_1203*) encoding ParB. The
158 strain did not show any growth or morphological defects (Fig. S1), indicating that the
159 ParB-sfGFP is a functional fusion. The fluorescence imaging of growing Z6 ParB-
160 sfGFP cells showed that the ParB-sfGFP exhibited several sub-cellular localisation
161 patterns (Fig. 1AB): 1) Multiple distinctive sfGFP foci were distributed throughout the
162 cytoplasm within a cell with noticeable variation in intensity. Interestingly, the *Z.*
163 *mobilis* ParB-foci did not exhibit an ordered spatial arrangement, in contrast to the
164 organized ParB positioning reported for several other bacteria including polyploid
165 species (17, 26-28). 2) There were often smeared signals around the foci, which
166 might suggest the spreading of ParB (29). 3) A larger focus with an intense signal
167 was observed at one of the cell poles. The polar focus exhibited, on average, more
168 than two-fold higher fluorescence intensity compared with non-polar foci. (Fig. 1C).
169 4) When the cells were washed with PBS, the fluorescent foci disappeared and the
170 signal became diffused throughout the cytoplasm (Fig. S2), indicating that the foci
171 were unlikely to be inclusion bodies.

172 We counted the distinctive fluorescent foci within cells using microbeJ (22) which
173 enables automated detection and quantitation of foci-signals within cells. However,
174 the number of detectable foci likely represents a lower-bound estimate of the *oriC*
175 numbers as *Z. mobilis* chromosomes appear to lack ordered spatial organization
176 within the limited cytoplasmic space, which could lead to co-localization of multiple
177 ParB-sfGFP foci (see Discussion). Despite this potential technical limitation, we
178 estimated a minimum chromosomal copy number of 5.6 +- 1.8 based on the
179 separated ParB-sfGFP foci (from evaluating 561 cells).



180

181 Fig. 1. ParB-sfGFP sub-cellular localisation in *Z. mobilis*. (A) Phase contrast (left) and fluorescent
182 images (middle, right) of growing Z6 ParB-sfGFP cells under standard growth conditions. For
183 visualising membrane, the cells were stained with FM4-64 (right panel). Scale bar, 5 µm. (B) ParB-
184 sfGFP detection in the *Z. mobilis* cells using microbeJ (22). Distinctive foci were detected from the
185 fluorescence images. Scale bar, 5 µm. (C) Box-and-whisker plot presenting ParB-sfGFP fluorescence
186 intensity of polar focus and foci detected from other cell regions. Biological replicates N = 3, total
187 measured foci N = 1063.

188

189 **Periplasmic Levan sucrase is toxic to *Z. mobilis* cells in the presence of**
190 **sucrose**

191 Having demonstrated that individual *Z. mobilis* cell contains multiple copies of
192 chromosome, we recognised that engineering the *Z. mobilis* genome requires an
193 efficient counter-selection system. In polyploid bacteria, the presence of multiple
194 chromosome copies could result in heterogeneous genotype during genome editing,
195 with wild-type and mutated genes coexisting on different chromosomes within the
196 same cell. Thus, positive selection strategies, such as antibiotic resistance markers,
197 might lack sufficient selection, because it allows the survival of cells that retained
198 unmodified, wild-type chromosome copies. In contrast, a negative selection
199 eliminates cells carrying unmodified chromosomes, efficiently selecting cells in which
200 all chromosomal copies altered.

201 Currently, the widely used method to delete gene in *Z. mobilis* is homologous
202 recombination-based method utilizing a suicide vector carries flanking region of the
203 targeted gene (30), particularly the method developed by Ral *et al.* which relies on
204 antibiotic resistance for the integration of the plasmid, and the loss of a GFP
205 fluorescence reporter for the excision of plasmid from the chromosome (20). This
206 powerful method has been previously used by us to successfully generate mutant
207 strains (31). However, we found that the last selection (excision of the integrated
208 plasmid) was not efficient, which resulted in obtaining mixture of cells that underwent
209 the excision and cells carrying the integrated plasmid. This was also observed in
210 another study that used the method (14). The high rate of false positive cells is
211 presumably due to the presence of multiple chromosome copies. In addition, the
212 method requires using time-consuming Fluorescence-Activated Cell Sorting (FACS).
213 Thus, we aimed to develop an alternative, practical negative selection method that

214 forces the excision event and allows simple isolation of cells that have the desired
215 genetic manipulation.

216 Although the *sacB* encoding levan sucrase was previously used as a counter-
217 selection in *Z. mobilis* (32) the use of levan sucrase was later questioned by the *Z.*
218 *mobilis* research community because the *Z. mobilis* genome encodes its own *sacB*
219 gene and can grow on sucrose as a sole carbon source. However, the native *Z.*
220 *mobilis* SacB was reported to be extracellular (33) while the toxicity of levan sucrase
221 manifests within the periplasm in Gram-negative bacteria (34). SignalP analysis (35)
222 predicts a periplasmic localisation for *Bacillus subtilis* SacB in Gram-negative
223 bacterial cells, but not for the *Z. mobilis* SacB consistent with the previous report
224 (33).

225 We next examined whether expressing the periplasmic $SacB^{BS}$ (*Bacillus subtilis*
226 SacB) could be toxic to *Z. mobilis* cells in the presence of sucrose and thus could
227 potentially serve as a counter-selector as in other Gram-negative bacteria. We
228 generated the *Z. mobilis* strain carrying $sacB^{BS}$ inserted into the chromosome at the
229 adjacent region of Z6_1449 (31), and tested growth in RM complex medium
230 supplemented with either 2% glucose or 5% sucrose. While the strain grew as wild-
231 type in the RM agar medium supplemented with 2% glucose, it failed to grow in the
232 presence of 5% sucrose (Fig. 2). This demonstrates the SacB-toxicity depends on
233 the cellular localisation of SacB and indicates that periplasmic $SacB^{BS}$ is applicable
234 for negative selection in *Z. mobilis*.

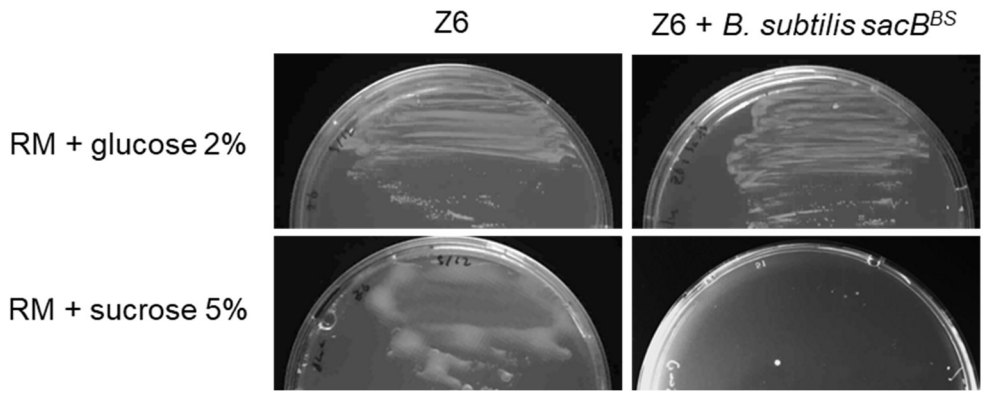
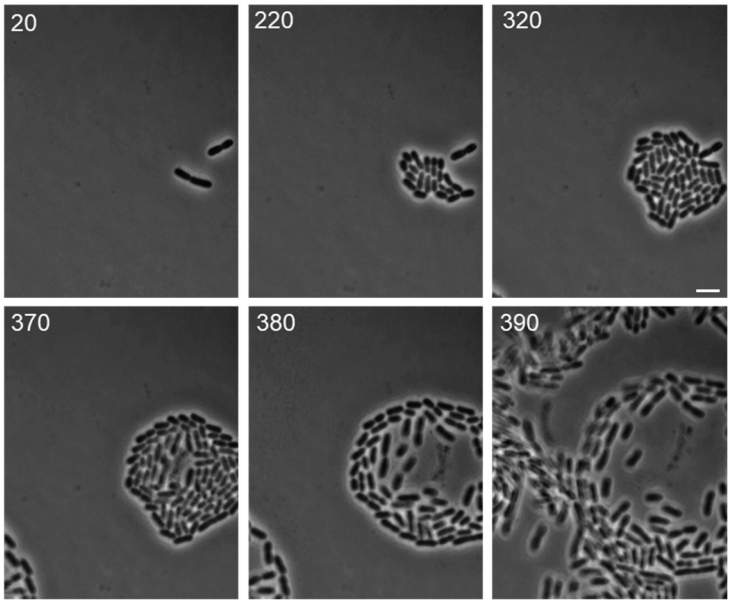


Fig. 2. Periplasmic $SacB^{BS}$ prevents growth of *Z. mobilis* cells in the presence of sucrose. (top) Both Z6 and Z6 + $SacB^{BS}$ strains grew on RM supplemented with 2% glucose. (bottom) Z6 exhibited lysing colonies on RM + sucrose 5% (left side) while Z6 + $SacB^{BS}$ strain did not grow at all in the presence of 5% sucrose (right side).

Sucrose in the growth medium triggers explosive lysis of wild-type *Z. mobilis* cell

Close inspection of the wild-type strain grown on RM agar-medium supplemented with sucrose indicated that the colonies displayed partial lysis (Fig. 2). The lysis is likely due to the activity of its native, extracellularly secreted SacB producing toxic levan polymer. Such stress-induced lysis might be a problem for a counter-selection system, as continuous stress might increase the likelihood of unwanted, secondary mutations during genome engineering. To observe the lysis at a cellular level, we performed time-lapse imaging on the wild-type strain Z6 growing on glucose or sucrose in the complex medium. While the majority of cells fed with sucrose grew well, we observed abrupt lysis of a few single cells during imaging (Fig. 3). The cytoplasmic content released from the cell pushed other cells on the agar-pad within 10 minutes during the imaging (Fig. 4, bottom panels). The explosive lysis was observed in other time-lapse videos, and typically affected only one cell within a

255 micro colony consisting of few hundred cells. This low level of lysis of Z6 in the
256 presence of sucrose presumably explains the slimy appearance of cells on the RM
257 agar-medium with sucrose (Fig. 2 left bottom) and the majority of cells remained
258 viable and continued to actively grow. Consistent with this, *Z. mobilis* cultures grown
259 in liquid medium containing sucrose exhibited no apparent growth defects as
260 previously reported (36, 37).

261 

262 Fig. 3. *Z. mobilis* cells growing in the presence of 5% sucrose. Images show that one cell exhibits
263 explosive lysis, releasing its cytoplasm content that washes away other cells on RM medium. (Time
264 point from top left to bottom right, 20, 220, 320, 370, 380, 390 minutes after the imaging started)
265 Scale bar, 5 μm.

266 **Periplasmic-SacB^{BS} counter selection is applicable in engineering the *Z.***
267 ***mobilis* genome**

268 Next, we applied the SacB^{BS} -based counter-selection system by integrating it into
269 the previously published method (20) by replacing the plasmid-excision step based
270 on FACS with sucrose-sensitivity selection. In this system, cells retaining the
271 integrated plasmid are killed in the presence of sucrose. Consequently, only cells in

272 which the plasmid has been excised from all chromosomal copies survive, ensuring
273 complete plasmid removal. Using this modified method, we readily obtained colonies
274 that appeared to have lost the targeted gene, as checked by PCR. To verify the
275 absence of wild-type gene in all chromosomes, we also analysed the whole genome
276 sequence of three independent strains (Δ*Z6_1449*, Δ*Z6_0810* and Δ*Z6_1479*). The
277 analysis confirmed the 100%, homozygous deletion of targeted gene without the
278 wild-type copy, and no additional mutations in other regions of the chromosomes. We
279 further utilised the SacB counter-selection system to introduce gene fusions or tags
280 into coding sequences (epitope or fluorescent tag) into the chromosomes. We
281 successfully obtained the strains with desired tags at the precise location in all
282 copies of chromosome, confirmed by whole genome sequencing (Z6_1449-His x 6-
283 FLAG). Table 1 summarises the generated strains and frequencies of obtaining the
284 desired genotype. On average, 29.8% of the mutant colonies (based on PCR
285 analysis) were obtained from the tested colonies by the sucrose selection, which we
286 found sufficiently effective and practical. Taken together, these results demonstrate
287 that the SacB-based counter selection is a reliable and efficient tool for engineering
288 the *Z. mobilis* genome.

289 Table 1, The summary of outcome of genotype after the excision of the plasmid
290 integrated into the wild-type strain Z6.

Introduced mutation/tag	**number of tested colonies**	**Genotype**		
		mixed	**wild-type**	**targeted**
Δ*Z6_1449*	3	1	1	1
Z6_1449-His x 6-FLAG	6	0	4	2
Z6_0812-mCherry	10	0	8	2
Δ*Z6_1449* C-ter truncation	11	0	10	1
Δ*Z6_0810*	6	0	5	1
Δ*Z6_0149*	12	1	9	2
Δ*Z6_1271*	22	0	15	7

ΔZ6_1369	24	0	15	9
Z6_1203-GFPsf	28	0	6	22
ΔZ6_1479	28	0	26	2
ΔZ6_0234	28	0	24	4

Discussion

This study adds new insights into the polyploidy and chromosomal organization of *Z. mobilis*. Our initial objective was to determine the chromosome copy number at the single-cell level using fluorescently tagged ParB that binds to *parS* site in the chromosome. However, this proved to be challenging due to the spatial organisation of chromosomes within the cell, and presumably led to underestimating the copy numbers because clustered foci were counted as single signal. In particular, the intense polar focus likely represents multiple *oriC* sites. In addition, *Z. mobilis* cells are approximately 1.1 - 1.4 µm wide, hence foci positioned above or below the focal plane might be missed when imaging of a single-plane. We aimed to visualise 3-dimentional imaging of the signals by Z-stacking of fluorescence imaging, but the foci were found to be dynamic, which made counting foci from stacked images unreliable (data not shown). Consequently, the copy numbers obtained from the imaging likely represent a lower-bound estimate. Consistent with this, the copies numbers obtained from the sfGFP signals were lower than those previously measured using quantitative PCR method (12).

The intense polar localization of ParB has been reported for other alpha-proteobacteria (28, 38) and might reflect a conserved feature of chromosome replication in this bacterial group, indicating that the observed intense foci might represent the ongoing replicating site.

313 The physiological benefits of polyploidy are not yet understood in *Z. mobilis*.
314 Previous work showed that the strain Z4 can grow under phosphorus-depleted
315 conditions, presumably by using its chromosome as a phosphorus source, which
316 might be a strategic advantage of being polyploid (39). Another proposed advantage
317 of polyploidy is to enhance genomic stability. A study on long-term co-cultivation of *Z.*
318 *mobilis* with *Saccharomyces cerevisiae* to induce genetic changes observed no
319 major mutations in the *Z. mobilis* chromosome, potentially due to the presence of
320 multiple chromosome copies that could facilitate reversion of mutations (40). On the
321 other hand, several studies have employed adaptive evolution to obtain *Z. mobilis*
322 strains with desirable traits (41-43) and as it remains unclear in some studies
323 whether the resulting populations were genetically homogeneous or contained mixed
324 genotypes, further examination in the context of the polyploid nature of *Z. mobilis*
325 might be needed.

326 In addition to advancing our understanding of chromosome organization, this study
327 also provides an additional genetic tool for genome engineering in *Z. mobilis*. We
328 showed that the frequently-used SacB counter selection is applicable to *Z. mobilis*,
329 and we implemented in the existing homologous recombination based method,
330 showing high frequency (29.8%) of obtaining the desired genotype. Recently, two
331 different counter-selection systems for *Z. mobilis* have been reported (44, 45). The
332 availability of multiple robust counter selection strategies should be advantageous for
333 genome engineering in *Z. mobilis*. Efficient counter-selection is not only useful for
334 homologous recombination–based approaches but also for recently developed
335 CRISPR-based systems, which needs reliable plasmid curing (46).

336

337

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References

1. Kalnenieks U. 2006. Physiology of *Zymomonas mobilis*: some unanswered questions. Adv Microb Physiol 51:73-117.
2. Swings J, De Ley J. 1977. The biology of Zymomonas. Bacteriol Rev 41:1-46.
3. Kalnenieks U, Balodite E, Strähler S, Strazdina I, Rex J, Pentjuss A, Fuchino K, Bruheim P, Rutkis R, Pappas KM, Poole RK, Sawodny O, Bettenbrock K. 2019. Improvement of Acetaldehyde Production in Zymomonas mobilis by Engineering of Its Aerobic Metabolism. Front Microbiol 10:2533.
4. He MX, Wu B, Qin H, Ruan ZY, Tan FR, Wang JL, Shui ZX, Dai LC, Zhu QL, Pan K, Tang XY, Wang WG, Hu QC. 2014. Zymomonas mobilis: a novel platform for future biorefineries. Biotechnology for Biofuels 7:101.
5. Wang X, He Q, Yang Y, Wang J, Haning K, Hu Y, Wu B, He M, Zhang Y, Bao J, Contreras LM, Yang S. 2018. Advances and prospects in metabolic engineering of Zymomonas mobilis. Metabolic Engineering 50:57-73.
6. Liu Y, Ghosh IN, Martien J, Zhang Y, Amador-Noguez D, Landick R. 2020. Regulated redirection of central carbon flux enhances anaerobic production of bioproducts in Zymomonas mobilis. Metabolic Engineering 61:261-274.
7. Wang Z, Wang X, Yan X, Yi H, He S, Zhang H, Zhou X, He Q, Yang S. 2024. Metabolic engineering of an industrial bacterium Zymomonas mobilis for anaerobic l-serine production. Synthetic and Systems Biotechnology 9:349-358.
8. Frohwitter J, Behrendt G, Klamt S, Bettenbrock K. 2024. A new Zymomonas mobilis platform strain for the efficient production of chemicals. Microbial Cell Factories 23:143.
9. Yan X, Bao W, Wu Y, Zhang C, Mao Z, Yuan Q, Hu Z, He P, Peng Q, Hu M, Geng B, Ma H, Chen S, Fei Q, He Q, Yang S. 2024. Paradigm of engineering recalcitrant non-model microorganism with dominant metabolic pathway as a biorefinery chassis. Nature Communications 15:10441.
10. Fuchino K, Chan H, Hwang LC, Bruheim P. 2021. The Ethanologenic Bacterium *Zymomonas mobilis* Divides Asymmetrically and Exhibits Heterogeneity in DNA Content. Appl Environ Microbiol 87.
11. Skerker JM, Leon D, Price MN, Mar JS, Tarjan DR, Wetmore KM, Deutschbauer AM, Baumohl JK, Bauer S, Ibáñez AB, Mitchell VD, Wu CH, Hu P, Hazen T, Arkin AP. 2013. Dissecting a complex chemical stress: chemogenomic profiling of plant hydrolysates. Molecular Systems Biology 9:674.

12. Fuchino K, Wasser D, Soppa J. 2021. Genome Copy Number Quantification Revealed That the Ethanologenic Alpha-Proteobacterium Zymomonas mobilis Is Polyploid. Frontiers in Microbiology Volume 12 - 2021.
13. Brenac L, Baidoo EEK, Keasling JD, Budin I. 2019. Distinct functional roles for hopanoid composition in the chemical tolerance of Zymomonas mobilis. Molecular Microbiology 112:1564-1575.
14. Braga A, Maia AB, Gomes D, Rodrigues JL, Rainha J, Rodrigues LR. 2025. Improving Fructooligosaccharide Production via sacC Gene Deletion in Zymomonas mobilis: A Novel Approach for Enhanced Prebiotic Production. Food and Bioprocess Technology 18:899-915.
15. Balodite E, Strazdina I, Martynova J, Galinina N, Rutkis R, Lasa Z, Kalnenieks U. 2019. Translocation of Zymomonas mobilis pyruvate decarboxylase to periplasmic compartment for production of acetaldehyde outside the cytosol. MicrobiologyOpen 8:e00809.
16. Xun ZPLR, Kwon,. Eilhann E., Jeong. Sang-Chul, & Jeon. Young Jae. 2015. Growth Characteristics of a Pyruvate Decarboxylase Mutant Strain of Zymomonas mobilis. Journal of Life Science 25:1290–1297.
17. Takacs CN, Wachter J, Xiang Y, Ren Z, Karaboja X, Scott M, Stoner MR, Irnov I, Jannetty N, Rosa PA, Wang X, Jacobs-Wagner C. 2022. Polyploidy, regular patterning of genome copies, and unusual control of DNA partitioning in the Lyme disease spirochete. Nature Communications 13:7173.
18. Jain IH, Vijayan V, O'Shea EK. 2012. Spatial ordering of chromosomes enhances the fidelity of chromosome partitioning in cyanobacteria. Proceedings of the National Academy of Sciences 109:13638-13643.
19. Zhang YH, Xue CM, Chen BT, Ouyang P, Ling C. 2025. Comparing three emerging industrial cell factories: Pseudomonas putida KT2440, Halomonas bluephagenesis TD01, and Zymomonas mobilis ZM4. Curr Opin Biotechnol 92:103255.
20. Lal PB, Wells FM, Lyu Y, Ghosh IN, Landick R, Kiley PJ. 2019. A Markerless Method for Genome Engineering in Zymomonas mobilis ZM4. Frontiers in Microbiology Volume 10 - 2019.
21. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature Methods 6:343-345.
22. Ducret A, Quardokus EM, Brun YV. 2016. MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. Nature Microbiology 1:16077.
23. Pédelacq J-D, Cabantous S, Tran T, Terwilliger TC, Waldo GS. 2006. Engineering and characterization of a superfolder green fluorescent protein. Nature Biotechnology 24:79-88.
24. Jalal ASB, Le TBK. 2020. Bacterial chromosome segregation by the ParABS system. Open Biol 10:200097.
25. Livny J, Yamaichi Y, Waldor MK. 2007. Distribution of centromere-like parS sites in bacteria: insights from comparative genomics. J Bacteriol 189:8693-703.
26. Shebelut CW, Guberman JM, van Teeffelen S, Yakhnina AA, Gitai Z. 2010. Caulobacter chromosome segregation is an ordered multistep process. Proceedings of the National Academy of Sciences 107:14194-14198.

27. Ucci AP, Martins PM, Lau IF, Bacci M, Jr., Belasque J, Jr., Ferreira H. 2014. Asymmetric chromosome segregation in Xanthomonas citri ssp. citri. Microbiologyopen 3:29-41.
28. Robalino-Espinosa JS, Zupan JR, Chavez-Arroyo A, Zambryski P. 2020. Segregation of four Agrobacterium tumefaciens replicons during polar growth: PopZ and PodJ control segregation of essential replicons. Proceedings of the National Academy of Sciences 117:26366-26373.
29. McLean TC, Le TBK. 2023. CTP switches in ParABS-mediated bacterial chromosome segregation and beyond. Current Opinion in Microbiology 73:102289.
30. Boismier EC, Aboulnaga EA, TerAvest MA. 2025. Zymomonas mobilis: bringing an ancient human tool into the genomic era. Current Opinion in Biotechnology 92:103257.
31. Fuchino K, Nalbant A, Gray J, Vollmer W. 2025. Peptidoglycan remodelling improves salt resilience of Zymomonas mobilis. bioRxiv doi:10.1101/2025.10.04.680478:2025.10.04.680478.
32. Xia J, Liu C-G, Zhao X-Q, Xiao Y, Xia X-X, Bai F-W. 2018. Contribution of cellulose synthesis, formation of fibrils and their entanglement to the self-flocculation of Zymomonas mobilis. Biotechnology and Bioengineering 115:2714-2725.
33. Gunasekaran P, Mukundan G, Kannan R, Velmurugan S, Aït-Abdelkader N, Alvarez-Macarie E, Baratti J. 1995. The sacB and sacC genes encoding levansucrase and sucrase form a gene cluster in Zymomonas mobilis. Biotechnology Letters 17:635-642.
34. Reyrat J-M, Pelicic V, Gicquel B, Rappuoli R. 1998. Counterselectable Markers: Untapped Tools for Bacterial Genetics and Pathogenesis. Infection and Immunity 66:4011-4017.
35. Teufel F, Almagro Armenteros JJ, Johansen AR, Gíslason MH, Pihl SI, Tsirigos KD, Winther O, Brunak S, von Heijne G, Nielsen H. 2022. SignalP 6.0 predicts all five types of signal peptides using protein language models. Nat Biotechnol 40:1023-1025.
36. Braga A, Maia AB, Rodrigues LR. 2025. Agro-industrial by-products valorization for fructooligosaccharide production with Zymomonas mobilis. Bioresources and Bioprocessing 12:110.
37. Bekers M, Laukevics J, Upite D, Kaminska E, Vigants A, Viesturs U, Pankova L, Danilevics A. 2002. Fructooligosaccharide and levan producing activity of Zymomonas mobilis extracellular levansucrase. Process Biochemistry 38:701-706.
38. Ebersbach G, Briegel A, Jensen GJ, Jacobs-Wagner C. 2008. A Self-Associating Protein Critical for Chromosome Attachment, Division, and Polar Organization in Caulobacter. Cell 134:956-968.
39. Brück P, Wasser D, Soppa J. 2023. One Advantage of Being Polyploid: Prokaryotes of Various Phylogenetic Groups Can Grow in the Absence of an Environmental Phosphate Source at the Expense of Their High Genome Copy Numbers. Microorganisms 11.
40. Fuchino K, Bruheim P. 2020. An assessment of serial co-cultivation approach for generating novel Zymomonas mobilis strains. BMC Research Notes 13:422.

41. Huang J, Wang X, Chen X, Li H, Chen Y, Hu Z, Yang S. 2023. Adaptive Laboratory Evolution and Metabolic Engineering of Zymomonas mobilis for Bioethanol Production Using Molasses. ACS Synthetic Biology 12:1297-1307.
42. Yan Z, Zhang J, Bao J. 2021. Increasing cellulosic ethanol production by enhancing phenolic tolerance of Zymomonas mobilis in adaptive evolution. Bioresource Technology 329:124926.
43. Phannarangsee Y, Jiawkhangphlu B, Thanonkeo S, Klanrit P, Yamada M, Thanonkeo P. 2024. Sorbitol production from mixtures of molasses and sugarcane bagasse hydrolysate using the thermally adapted Zymomonas mobilis ZM AD41. Scientific Reports 14:5563.
44. Zheng Y, Fu H, Chen J, Li J, Bian Y, Hu P, Lei L, Liu Y, Yang J, Peng W. 2023. Development of a counterselectable system for rapid and efficient CRISPR-based genome engineering in Zymomonas mobilis. Microbial Cell Factories 22:208.
45. Binan G, Yalun W, Xinyan W, Yongfu Y, Peng Z, Yunhaon C, Xuan Z, Chenguang L, Fengwu B, Ping X, Qiaoning H, Shihui Y. 2024. Efficient genome-editing tools to engineer the recalcitrant non-model industrial microorganism Zymomonas mobilis. Trends in Biotechnology 42:1551-1575.
46. Chen M, Huang Y, Zheng Y, Wu B, He M. 2023. The escape of CRISPR-mediated gene editing in Zymomonas mobilis. FEMS Microbiology Letters 370.