Increased salt tolerance in *Zymomonas mobilis* strain generated by adaptative evolution

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Abstract

Background
Ethanologenic alphaproteobacterium *Zymomonas mobilis* has been acknowledged as a promising biofuel producer. There have been numerous efforts to engineer this species applicable for an industrial-scale bioethanol production. Although *Z. mobilis* is robustly resilient to certain abiotic stress such as ethanol, the species is known to be sensitive to saline stress at a mild concentration, which hampers its industrial use as an efficient biocatalyst. To overcome this issue, we implemented laboratory adaptive evolution approach to obtain salt tolerant *Z. mobilis* strain.

Results
During an adaptive evolution, we biased selection by cell morphology to exclude stressed cells. The evolved strains significantly improved growth and ethanol production in the medium supplemented with 0.225 M NaCl. Furthermore, comparative metabolomics revealed that the evolved strains did not accumulate prototypical osmolytes, such as proline, to counter the stress during their growth. The sequenced genomes of the studied strains suggest that disruption of ZZ6_1149 encoding carboxyl peptidase was likely responsible for the improved phenotype.

Conclusions
The present work successfully generated the strains able to grow and ferment glucose under saline condition that severely perturbs parental strain physiology. Our approach to generate strains, cell shape-based diagnosis and selection, might be applicable to other kind of strain engineering in *Z. mobilis*.

Background
Biofuel is a renewable clean energy source derived from biomass. With increasing environmental concerns about the use of fossil-dependent fuels, development of sustainable biofuel-refinery has lately attracted public attention. Currently, the baker’s yeast *Saccharomyces cerevisiae* is regarded as the best promising bioethanol producer [1]. However, an alternative candidate, facultative anaerobic alphaproteobacterium *Zymomonas mobilis* is an efficient ethanol producer exhibiting
several attractive physiological features surpassing *S. cerevisiae*, such as high specific rate of sugar uptake, relatively small genome size, low biomass production, a capacity to fix nitrogen gas [2-5], and superior ethanol productivity [4, 6]. Its efficient homo-ethanol fermentation, mediated by Entner-Doudoroff (ED) pathway where up to 50% of total cellular soluble protein is involved as catalytic enzymes, coupled to active pyruvate carboxylase and two alcohol dehydrogenases, catabolizes simple sugars efficient and produces ethanol nearly at theoretical maximum yield in *Z. mobilis* [4].

The potential of *Z. mobilis* culture in industrial use is not strictly restricted in converting sugar to ethanol. It has been emerging as a producer of different high-value chemicals [7-9].

To expand its potential as a biocatalyst, increasing stress tolerance is critical in actualizing *Z. mobilis* based biorefinery. *Z. mobilis* cells are intrinsically tolerant of very high concentration of ethanol (>10%, v/v) and broad pH range (3.5–7.5) [10]. However, ubiquitous inorganic salt sodium chloride inhibits *Z. mobilis* growth at mild level [10, 11]. Addition of 10 g/L NaCl in the complex growth medium, a concentration commonly used in bacterial growth medium, significantly perturbed the growth and ethanol production of *Z. mobilis* [11]. *Z. mobilis* also exhibits abnormal cell shape under salt condition, by elongating rod/ovale shape and forming long filamentous structure with a bulged swollen pole [11]. Considering that *Z. mobilis* grows well in the presence of high amount of glucose [7], osmotic pressure per se may not be the sole cause, but ionic disturbance by salt likely attributes to the poor growth.

Inorganic ions are potential inhibitor in lignocellulosic hydrolysate, environmentally and economically appealing substrate for biofuel production. Also, the common salt NaCl may be found in different range of renewable industrial feedstock [12]. De-salination before fermentation is not welcomed due to its high cost. Thus, *Z. mobilis* strain that can grow and ferment under salt condition is on demand. Nevertheless, only several studies have tacked this problem so far. Previously, a systematic approach introducing transposon mutation in *Z. mobilis* identified that mutation in *himA* increased tolerance to saline stress [13]. Although the mechanism of action was not clarified, the *himA* mutant strain exhibited improved growth and ethanol production under saline condition. The Na⁺/H⁺ antiporter in strain ZM4 was also identified as an important transporter in sodium ionic stress [14]. Strains
obtained from these works showed promising improvements, yet, to a mild degree or under limited condition. Interestingly, it was suggested the potential biological significance of respiratory chain – enigmatic, low-energy coupled aerobic respiration in Z. mobilis might be important for maintaining low reduced/oxidized form of co-factor Nicotinamide Adenine Dinucleotide (NAD) upon saline stress [15].

General bacterial stress response to external high osmolality is to accumulate low molecular mass compatible solutes, through transporting and biosynthesis [16]. This type of response in Z. mobilis was previously reported, showing a relative increase of several metabolites under saline condition [17]. On the other hand, Kohler et al. reported that Z. mobilis genome misses most of loci encoding compatible solute transport proteins such as Kdp complex and BetS, implying that the transporting mechanism is not involved in this species to encounter stress [18]. In addition, accumulation of sorbitol was suggested to be important in osmotic stress response in Z. mobilis [19], however, this regulation was not confirmed in another study [15], exhibiting the discrepancy. Taken all together, these studies suggest that de novo synthesis of molecule appears to be critical for the adaptation in Z. mobilis.

For improving Z. mobilis cells against external abiotic stress, several studies adopted top-down or forward approach. These methods includes an error-prone PCR based mutagenesis [20], an adaptive laboratory evolution method [21-23], genome shuffling [24] and a transposon approach [13]. In the present study, we improvised adaptive laboratory evolution approach to generate saline resilient strains. The obtained strains successfully improved cellular growth and ethanol production under saline condition. Furthermore, we examined the promising strains by quantitative metabolomics and whole genome sequencing, in order to understand what mechanism rendered the salt tolerant phenotype of strains.

Results And Discussion
Adaptive laboratory evolution to generate salt-resilient strain
To generate Z. mobilis strains that can grow and ferment under saline stress condition, we adopted an adaptive laboratory evolution strategy. The approach was previously employed by Wang et al.,
however the *Z. mobilis* culture with excessive NaCl concentration was not viable for long term due to a toxic effect, and the approach was not successful in generating resilient strain [13]. Therefore, we introduced a bias in the serial transfer on a following ground, to direct evolution preferable to our goal (Fig. 1).

Previously, it was shown that salt condition induces filamentation of *Z. mobilis* cell [11]. We observed the filamentation in our experimental setting, using our standard complex medium supplemented with 0.225 M NaCl (Fig. 2). Interestingly, almost all cells exhibited an abnormal bulge at single cell pole, with variant cell length and width (Fig. 2 top right). These cells were found as thick sedimentation in the fully-grown culture (Fig. 1), however, we did not observe any floc formation which was shown to be beneficial for stress resistance in *Z. mobilis* [25]. Based on cell shape and growth profile (Fig. 2, Fig. 3), we speculated that the filamentous shape with a bulged pole is a consequence of stress, rather than an adaptation to environment. We then thought the filamentation to be exploited as a biomarker to identify the stressed cells that should be avoided for serial transfer. Although the sedimentation might have involved other factors, microscopic observations led us to conclude that filamentation of cells facilitated a formation of sediment.

Following the rationales, we performed lab-directed evolution with a bias in transfer. In practical, we collected cells only from an upper layer in the fully-grown culture as an inoculum for next round (Fig. 1). Zm6 strain was evolved in the complex medium supplemented with 0.2 M NaCl for 13 transfers, then continued in the medium with 0.225 M NaCl for another 8 transfers. After about first 10 transfers, it was apparent from turbidity of cultures that the strains improved growth. Strains after 13th transfer was designated as KFS1 and after 21st transfer as KFS2. To clarify if the improved growth was due to temporal physiological adaptation without genetic mutation or stable phenotype arose from mutations, we performed a pilot experiment. The evolved strain culture without adaption, i.e., grown in the medium without additional salt, was used as an inoculum for measuring growth profile under salt condition (0.225 M NaCl). We observed the improved growth, excluding the possibility of transient adaptation and showing that mutation in the genome was the cause.

Evolved strains exhibited two characteristics in cell shape under salt condition. The strains less
frequently formed bulged pole than parental strain did (Fig. 2, bottom panels), indicating that the cells were less stressed under salt condition. The cells were also found fragmented or long filament shaped which was rather contrary to what we expected (Fig. 2).

Characterization of evolved strains
Next, we characterized phenotypes of presumably evolved strains by measuring growth curve, glucose consumption and ethanol production by strains in the presence of salt. It is to be noted that used inoculum for the culture was not adapted to salt condition. (See material and method).
As shown in the figure 3, parental strain Zm6 did not consume all available glucose in the medium with salt, failing completion of fermentation due to an arrest of growth. In a sharp contrast, the evolved strains drastically improved growth and ethanol production under the same condition (Fig. 3).
Remarkably, final biomass of evolved strain was about 2.5 and 2.7 times higher than that of Zm6, respectively for KFS1 and KFS2 (Fig. 3). Total ethanol production by KFS1 and KFS2 was also significantly improved, 2.74 and 2.69 times higher than by parental strain. (Fig. 3). It is to be noted that the final biomass (CDW mg/mL) and ethanol yield [EtOH(g)/Glucose(g)] by evolved strains under salt condition was close to those by parental strain under non-salt condition.
To characterize the strains further, growth profiles of all strains in the medium without supplement of salt were recorded (Fig. 3). Both evolved strains showed slight retarded growth and ethanol production under non-salt condition, and their final biomass of evolved strains was significantly lower than that of parental strain. This was somewhat expected by us, considering that the improvement of growth in salt medium was drastic and likely involved physiological alternation. However, final ethanol production by all strains was nearly same (Fig. 3), showing that final ethanol production per cell dry weight was, interestingly, higher in the evolved strain. This implies that cellular activity was spatially more ‘packed’ in the evolved strain. Along with this line, we observed that the evolved cells exhibited smaller cell size than parental strain, as shown by light microscopic images (Fig. 4).

Quantitative Metabolomics
Bacterial osmotic response involves accumulation of osmoprotectant to counter external osmolality, to maintain turgor. Proline and betaine-glycine are examples of well characterized osmoprotectant in
several bacteria [26]. Such a response, accumulating specific compounds as osmolyte, may result in dynamic metabolic change and could perturb production of desirable compounds by \textit{Z. mobilis}. To determine if any metabolomic adjustments played a significant role in acquired resilience in the evolved strains, we performed quantitative targeted metabolic profiling of central carbon metabolites and free amino acids in all strains.

Our initial goal was to obtain intracellular concentration for a comparative analysis. The challenge here was that cellular volume was highly heterogeneous in all strains under salt condition (Fig. 2), hindering intracellular concentration measurements that require defined cell volume. Therefore, we first normalized metabolite abundance in each strain by cell dried weight (Fig. 5). The normalized metabolites abundance was compared between strains and conditions, as shown by the heatmap of log2 fold change in the Fig. 5. From fixed weight of cell extract, Zm6 cells without stress (Zm6 NS) generally comprised larger pool of the metabolites than Zm6 cells under salt condition (Zm6 S) (Fig. 5, left panel). Interestingly, several free amino acids including proline are the only metabolites found upregulated under salt condition per fixed dry weight, although mildly. Similarly, extracts from evolved strains under salt condition (KFS1 S, KFS2 S) showed smaller pool size of the metabolites than from Zm6 NS (Fig. 5, middle and right panel).

Next, we stained membrane of \textit{Z. mobilis} cells with a staining dye Fm4-64 to observe if there is a compartmentalization within cell. We observed no septum formation nor membrane organelle in the bulged Zm6 cells (Fig. 6). Apparently, salted Zm6 single cell volume was much larger than that of rod shape Zm6 under non-salt condition (Fig. 2, Fig. 6).

According to previous studies, Protein, DNA and RNA are the main component constituting about 70-80% of weight in bacteria [27]. It is less likely that slow growing filament (Zm6 S) possessed more dense macro molecules per fixed cell volume than actively growing small cell (Zm6 NS). We therefore speculated that Zm6 S is expected to have larger cell volume per fixed amount of CDW than that of Zm6 NS. This further leads to that the log2 ratio of Zm6 S/ Zm6 NS in Fig. 5, normalized by CDW, should decrease when intracellular concentration is deployed. Thus, our metabolomics data suggest that Zm6 cells did not drastically accumulate central metabolites or free amino acids during the stress
response. Most of ED pathway metabolites and nucleosides in Zm6 S was drastically downregulated, coinciding with low growth and slow glucose uptake. Moreover, canonical osmoprotectant, for example proline, was not found significantly accumulated either. Although, it was not completely excluded that other metabolites might have been upregulated during the stress response. KFS1 and KFS2 under saline condition exhibited heterogeneous cell shapes, ranging from fragmented cells to long extended cells (Fig. 2). Membrane staining showed that cells could produce septum at several locations, unlike Zm6. Nevertheless, KFS1 cells did not complete division and instead formed long filament, and KFS1 S cell compartment size was found overall larger than that of Zm6 S (Fig. 6). Similar to the case of comparison of Zm6 extract between saline conditions, bigger volume and slower growth of KFS1 S and KFS2 S to Zm6 NS imply that actual intracellular concentration ratio of KFS1 or KFS2 S to Zm6 NS is smaller than the log2 ratio depicted in Fig. 5. This suggests that evolved strains did not accumulate osmolytes to counter the stress either. To further understand the resilient mechanism in evolved strains, we measured intracellular ratio of reduced/oxidized form of NAD cofactor (NADH/NAD) ratio by an enzymatic assay. Malate dehydrogenase and oxoglutarate dehydrogenase complex are not encoded in Z. mobilis genome [3, 28], which influences regeneration of NAD. Previous works suggested that maintaining low NADH/NAD ratio appear to be important in response against salt and acetic acid stress, to sustain glycolysis in the ED pathway that requires oxidized NAD [15, 29]. To see if the redox regulation of co-factor conferred the resilience, we examined the NADH/NAD ratio in the strains. The analysis showed that stress increased the ratio in Zm6 (Table 1), as it was previously shown [15]. Interestingly, upregulation of NADH/NAD ratio under saline condition was also observed in evolved strain as well, within similar range of shift to Zm6. These data indicate that modulation of NADH/NAD was not a part of resilience mechanism in the evolved strains. Although the modulation of ratio is vital in general Z. mobilis stress response, as shown by previous studies, the resilience to salt stress in the evolved appeared to be mediated by a separate mechanism. Identification Of Gene Loci Next, we sought to identify what mutations in genome brought the phenotype in evolved strains. The
whole genomes of parental and evolved strains were sequenced and aligned against the reference genome [28]. The analysis showed that our lab stock Zm6 strain possesses several mutations (Table S2). These mutations likely arose during previous laboratory practices. We found several mutations only arose in evolved strains (Table. 2). A disruptive insertion in ZZ6_1449 coding carboxyl protease (CTP) was among them. CTP is found in all kingdom of life and mainly cleaves serine or lysine nearby at C-terminus of substrate. In bacteria, it has been shown that mutation in CTP caused alternation in cell envelop and higher sensitivity to antibiotics [30] and osmotic down-shift in Escherichia coli [31]. In Pseudomonas aeruginosa, disruption of CTP results in impaired growth in medium with low salt [32]. The phenotype in E. coli and P. aeruginosa is to some extent consistent with our results from growth profile. Other three point-mutations only found in KFS1 and KFS2 are possibly irrelevant with stress resilience, based on the annotated function. We found a point-mutation only found in KFS2, and an annotation of mutated gene is not available from the database. There are mild differences between KFS1 S and KFS2 S in morphology and growth, and it cannot be excluded that the mutation in the uncharacterized gene conferred extra resilience in KFS2. Together with available literature, it strongly suggests that disruption of ZZ6_1449 was mainly responsible for the improvement of growth. To confirm that the phenotype was due to the disruption, we attempted to perform complementation test in evolved strains. However, we did not obtain the construct due to technical challenges in cloning, and the test was not performed.

Conclusion
The present study successfully generated strains that can grow and produce ethanol under salt condition. Our approach was to bias selection by sedimentation nature of cells, which might be utilized in other kind of strain improvement in Z. mobilis. Interestingly, the evolved strains did not adapt to saline environment by adjusting known osmolyte concentration or modulation of intracellular NADH/NAD ratio. Genome sequencing revealed that disruption of ctp was likely responsible for the improvement by altering cell envelope profile. Further elucidation of evolved strains might shed light on mechanistic understanding of salt stress response in Z. mobilis.

Materials And Method
Adaptive laboratory evolution
Z. mobilis ATCC 29191 was used as a parental strain for laboratory evolution. Zm6 was cultivated in a complex growth medium containing glucose (20 g/L), yeast extract (5 g/L), NH₄SO₄ (1 g/L), KH₂PO₄ (1 g/L), MgSO₄ (0.5 g/L), with supplemental NaCl (final concentration; 0.2 M – 0.225 M) to generate salt tolerant strain. The complex medium was flashed by nitrogen gas filtered through sterilized 0.2 µm Supor® (polyethersulfone) membrane (PALL) prior to use. 12 mL of anaerobic Zm6 culture was grown at 30 °C in a tightly capped 15 ml falcon tube with shaking at 200 rpm. 30 µL of inoculum was transferred from previous round of culture for evolution. Transfer of cells to fresh medium was performed when cells reached stationary phase. First 13 transfers were done in the medium with 0.2 M salt, generating KFS1 strain. The rest of evolution was performed in the medium with 0.225 M for another 8 transfers, generating KFS2 strain.

Characterization Of Evolved Strains
To analyze cellular growth in salt condition, overnight culture was inoculated into anaerobic standing cultures and incubated at 30 °C. It is to be noted that used inoculum was grown in the same growth medium without additional NaCl, thus the inoculated cells were not adapted to salt condition. The optical density (absorbance at 600 nm) of culture was measured using a spectrophotometer (VWR). The cell dry weight was measured as follows; growing cells were centrifuged for 5 minutes (4500 x g) and washed in phosphate buffered saline (PBS) solution (OXOID). The washed cells were transferred to a foil cup package (VWR) and dried in oven at 90 °C till the solutions got completely dried. The dried cells on foil were weighed and subtracted by the tare weight of foil and dried PBS. Figure 3 was made using measured OD₆₀₀ and conversion factor CDW [g/L]/OD₆₀₀ (table S3).

Determination of glucose consumption and ethanol production in spent media was performed using Waters 2695e Alliance HPLC (Waters) with Hi-plex column (300 x 7.7 mm, Agilent) under a running condition; 0.05M sulfuric acid as mobile phase at a flow rate of 0.8 mL/min. MQuant® Glucose test kit (Merck) was also tested for an estimation of glucose presence (10–500 mg/L).

Metabolomics
Cell extractions were prepared as described in [33, 34]. Central carbon metabolites and nucleoside-phosphate were analyzed by capillary ion chromatography [33] coupled to tandem mass
spectrometry, TQ-XS (Waters). Free amino acids were first derivatized by Edman’s reagent [35]. Derivatized samples were analyzed by UPLC (waters) coupled to TQ-XS as described in [36]. All metabolites measurements were corrected by isotopic dilution method as described in [36]. Determination of NADH/NAD ratio was performed using an enzymatic assay kit, following the instruction supplied by the manufacturer (Sigma). Briefly, growing culture was centrifuged (3 min, 4500 x g) and supernatant was carefully discarded. Pellets were quickly frozen by liquid nitrogen for storage at -80 °C. Upon the measurements, cells were thawed on ice and resuspended in the extraction buffer supplied from the kit. Absorbance at 450 nm was measured every 20 minutes for 4 hours using 96 well-plates in plate-reader spark 20M (Tecan).

**Fluorescent Microscopy And Image Analysis**

Live cells were observed using a Zeiss Axio Imager Z2 microscope. The images were captured by Axiocam MR R3 (ZEISS) and analyzed by ZEN 2.3 pro software (ZEISS). For staining membrane, growing cultures were centrifuged (4500 x g) for 5 minutes and the pellet was resuspended in phosphate buffered saline solution. FM4-64 (Thermo Fisher Scientific) was then added at final concentration of (20 µg/ml) and incubated for 15 minutes. Cells were then washed in PBS again and mounted on PBS agarose-pad (1% w/v).

**Whole Genome Sequencing**

Whole genome of the studied strains was sequenced by GATC re-sequencing service (INVIEW Genome sequencing). Total DNA extraction was performed combining a lysing method [37] and D-neasy blood tissue kit (Qiagen). Briefly, lysozyme treatment was performed as follows. Overnight culture was centrifuged (20000 x g) for 1 minutes, and pellets were washed in TE buffer (10 mM Tris-Cl pH7.5, 1 mM EDTA) and centrifuged again. The pellets were resuspended in lysozyme solution and incubated for 30 minutes at 37 °C. (Lysozyme solution; lysozyme 50 mg/ml in buffer containing 10 mM Tris-Cl pH 8, 2.5 mM EDTA, 20 mM NaCl). This procedure was followed by proteinase K incubation (20 mg/ml, 1hour at 50 °C) and the rest of procedures followed the instructions provided the manufacturer.

Concentration and purity of genomic DNA was determined using nanodrop one (thermo scientific).

**Abbreviations**

ED
Entner-Doudoroff
NAD
Nicotinamide Adenine Dinucleotide
CDW
Cell Dry Weight
OD
Optical Density
HPLC
High Pressure Liquid Chromatography
UPLC
Ultra-Performance Liquid Chromatography
SD
Standard Deviation
WGS
Whole Genome Sequencing

Declarations
Competing interests
The authors declare that they have no competing interests.

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Tables
Due to technical limitations, Tables 1-2 are provided in the Supplementary Files sections.

Captions:
Table 1. NADH/NAD ratio in growing Zm6 and evolved strains under non-saline or saline conditions. The NADH/NAD ratio in all studied strains were measured using an enzymatic assay kit. Three biological replicates were measured for each strain under saline or non-saline condition. SD stands for standard deviation.

Table 2. List of mutation only found in the evolved strains. The whole genome sequence of lab stock Zm6 strain and the salt tolerant strains KFS1 and KFS2 were sequenced and compared to the reference genome [28]. Position of the mutated base is according to the reference genome. The mutations only found in KFS1 and KFS2 are shown in this table, while the common mutations found in all strain, derived from our lab stock Zm6 strain, is shown in Table S2.

Figures
Figure 1

Morphology-biased serial transfer for generation of salt resilient Z. mobilis strains. The picture shows the fully grown Zm6 culture in the complex medium supplemented with NaCl 0.225 M. The sediments of Zm6 Z. mobilis cells at bottom, constituting clumps of bulged/filamented cells, were observed under microscope. In an upper layer of the medium (pointed by gray arrow), shorter filamented cells resided, and this part of culture was transferred as an inoculum for next round of evolution.
Figure 2

Improved morphology of evolved Z. mobilis strains under salt condition. All images were taken by a phase contrast microscope. Left top; growing Zm6 in the complex medium without addition of salt. Right top; growing Zm6 in the medium containing 0.225 M NaCl, showing abnormal bulged shape at a pole of elongated cell. Left bottom; growing strain KFS1 in the complex medium with 0.225 M NaCl, showing long filamentation of cells with occasional bulged shapes. Right bottom; growing strain KFS2 in the complex medium with 0.225 M NaCl, exhibiting shorter filamentation and less occurrence of bulged cell pole comparing to KFS1. Scale bar applies to all images; 50μm.
Figure 3

Fermentation profiles of studied strains under salt or non-salt condition. The plots show growth curve, glucose consumption and ethanol production under saline (0.225 M NaCl) condition (left panel) or non-salt condition (right). Error bar represents standard deviations of 3 independent measurements. KFS1 and KFS2 exhibited improved growth and fermentation capacity under saline condition, while these strains grew mildly-slower than parental strain did under non-salt condition.
Cell sizes are reduced in the evolved strains under non-saline condition. Histogram (top two and left bottom panel) shows the distribution of cellular length of growing Zm6 (top left), KFS1 (top right) and KFS2 (left bottom) under non-saline condition (N = 150 for Zm6, N = 133 for KFS1 and KFS2). Cells were grown in the complex medium under anaerobic condition until OD600 of the cultures reached around 1 in all strain. Cells were then mounted on agar-pad and imaged by a phase contrast microscope. Kernel density was plotted using bandwidth of 0.289 for all histograms. Phase contrast images of each strain under non-saline condition (left bottom, strain is indicated in the image) is shown with a scale bar 10 μm applied for all images. Small table in the right bottom displays the mean value of cellular length (μm) in each strain. SD stands for standard deviation.

**Table 1**

| Strain | Mean ± SD (μm) |
|--------|----------------|
| Zm6    | 3.31 ± 0.84    |
| KFS1   | 2.95 ± 0.81    |
| KFS2   | 2.76 ± 0.69    |
Figure 5

Heatmap representing the log2 fold change of measured metabolites between different conditions or strains. Left column represents ratio of Zm6 under non-salt condition (Zm6 NS) versus Zm6 under salt condition (Zm6 S). Middle column shows the ratio Zm6 NS versus KFS1 under salted condition (KFS1 S) and right column shows the ratio Zm6 NS versus KFS2 under salted condition (KFS2 S). The numbers are log2 fold change of metabolites abundance. A table of absolute abundance (pmol/CDW mg) and abbreviation of metabolites are found in supplementary material (Fig. S1 and Table S1). Colour gradient represents a scale of log2 value. NS in sample designation stands for growth condition without a supplement of salt in medium while S stands for condition with 0.225 M NaCl in the medium.
Membrane staining of Zm6 and KFS1 strains growing under salt condition. Growing Zm6 and KFS1 under salt condition (NaCl 0.225 M) was stained by Fm4-64 at concentration of 20 μg/ml for 15 minutes for membrane visualization. The dye was washed prior to mounting on the agar-pad for fluorescent microscopy. Imaging revealed that bulged Zm6 (top panels) did not show any septa nor membrane compartment inside of cells. KFS1 (bottom panels) occasionally formed very long filaments over 50 μm without any septa within cell. Locally frequent septa formation was found in some KFS1 cells, as pointed by white arrows. These suggest that septation was not tightly controlled in the strain under salt condition. Red; Fm4-64 fluorescent signal. Scale bar; 10 μm.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.
Table2.tif
Table1.tif
Supplementaryfiles.docx