

# Discovery of Ethanol-Responsive Small RNAs in *Zymomonas mobilis*

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*Zymomonas mobilis* is a bacterium that can produce ethanol by fermentation. Due to its unique metabolism and efficient ethanol production, *Z. mobilis* has attracted special interest for biofuel energy applications; an important area of study is the regulation of those specific metabolic pathways. Small RNAs (sRNAs) have been studied as molecules that function as transcriptional regulators in response to cellular stresses. While sRNAs have been discovered in various organisms by computational prediction and experimental approaches, their discovery in *Z. mobilis* has not yet been reported. In this study, we have applied transcriptome analysis and computational predictions to facilitate identification and validation of 15 novel sRNAs in *Z. mobilis*. We furthermore characterized their expression in the context of high and low levels of intracellular ethanol. Here, we report that 3 of the sRNAs (Zms2, Zms4, and Zms6) are differentially expressed under aerobic and anaerobic conditions, when low and high ethanol productions are observed, respectively. Importantly, when we tested the effect of ethanol stress on the expression of sRNAs in *Z. mobilis*, Zms2, Zms6, and Zms18 showed differential expression under 5% ethanol stress conditions. These data suggest that in this organism regulatory RNAs can be associated with metabolic functions involved in ethanol stress responses.

High tolerance to ethanol is a desirable feature for ethanologenic strains used in industry. Given that ethanol is toxic to cells by inhibiting cell growth and metabolism, production of ethanol itself represents a bottleneck for the industrial use of biological systems (1, 2). *Zymomonas mobilis* is a Gram-negative bacterium that can efficiently produce ethanol from several carbon sources, including glucose, fructose, and sucrose, via the Entner-Doudoroff pathway (3). In addition, *Z. mobilis* maintains cell viability anaerobically when yielding high levels of ethanol (4). In fact, several reports have indicated that the presence of oxygen during fermentation affects ethanol production due to increased number of inhibitors (e.g., acetaldehyde and acetate) under aerobic conditions (5, 6). On the other hand, anaerobic growth of *Z. mobilis* can facilitate rapid glucose consumption with an increase in ethanol production relative to aerobic fermentation (5, 7).

*Z. mobilis* has a number of desirable characteristics that make it attractive as a biofuel organism (8). For instance, *Z. mobilis* can efficiently produce ethanol up to 12% (wt/vol) from carbohydrates at a higher rate and a 3- to 5-fold-higher yield than yeast (9). In addition to its high ethanol-producing capability with relatively low biomass, its rates of sugar uptake and processing are also high. Other advantages include the following: (i) *Z. mobilis* can tolerate up to 16% (wt/vol) ethanol, (ii) *Z. mobilis* is easy to handle for genetic manipulation and therefore amenable for developing recombinant strains with enhanced ethanol productivity, and (iii) the complete genome sequence of *Z. mobilis* is available for metabolic engineering (3, 10–12).

An intriguing aspect of *Z. mobilis* is the potential shifts in metabolism that likely occur as the organism transitions from high to low oxygen, where it is the most efficient at accumulating ethanol. In this work, we wanted to examine the potential role of regulatory small RNAs (sRNAs) in this process. These regulators are relatively short in prokaryotes (~50 to 300 nucleotides) and are not translated (13, 14), although a possibility is that they produce small (functional or nonfunctional) peptides. Therefore, sRNAs represent a subset of noncoding RNAs that can be both activators and repressors for regulating proteins and mRNAs via a variety of mechanisms. For instance, (i) antisense sRNAs affect translation

and mRNA stability of the complementary target gene, and (ii) *trans*-acting sRNAs regulate mRNAs by imperfect base-pairing with distal mRNA targets (15–17). sRNAs have been known to regulate various metabolic pathways under cellular stress conditions such as oxidative stress, ethanol, and temperature or pH change (18–20). When cells encounter environmental changes, regulatory sRNAs help to modulate gene expression by optimizing cellular metabolism for survival. Our motivation in this work was rooted by the ubiquitous discovery and validation of these regulatory elements in bacteria using many computational and experimental strategies (14, 21–23). Interestingly, recent data have shown higher expression of Hfq under anaerobic conditions in *Z. mobilis*, with higher ethanol production than under aerobic conditions (4). Hfq is a conserved bacterial Sm-like family of RNA-binding proteins, particularly in Gram-negative bacteria, that can bind sRNAs and their target mRNAs to direct functionality (24, 25). In addition, Hfq has been shown to play an important role in tolerance to multiple biomass pretreatment inhibitors such as acetate, vanillin, and furfural (26) in *Z. mobilis*. Collectively, these findings supported our initial hypothesis regarding the possibility that sRNAs play important mechanistic roles under differential oxygen (and thereby ethanol) conditions in this bacterium.

The study of potential sRNA regulation in the context of bacterial strains that are capable of producing and tolerating high levels of biofuels (and precursors) dates back to previous studies. For instance, small RNAs have been confirmed in *Clostridium acetobutylicum*, another important strain in the production of ac-

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etone and biobutanol from carbohydrates (27–29). In the case of *Z. mobilis*, although its genome has been completely sequenced (30), most research has focused on describing membrane composition, understanding patterns of gene expression, and characterizing lipid composition. In this study, we focused on investigating the potential presence of regulatory sRNAs in *Z. mobilis*. We furthermore characterized the expression of these newly uncovered RNA elements under anaerobic and aerobic conditions (known to result in differential levels of ethanol accumulation).

## MATERIALS AND METHODS

**Strains and culture condition.** *Zymomonas mobilis* ZM4 (ATCC 31821) was cultured in RM medium (glucose, 20.0 g/liter; yeast extract, 10.0 g/liter;  $\text{KH}_2\text{PO}_4$ , 2.0 g/liter [pH 6.0]) (55) at 30°C (pH 6.0). A single colony was inoculated into 5 ml of RM medium and cultured aerobically or anaerobically at 30°C overnight. A 1/100 of initial culture was added to 1 liter of prewarmed RM broth and then cultured overnight at 30°C with shaking at 150 rpm. The inoculum was added to each culture so that the initial optical density at 600 nm ( $\text{OD}_{600}$ ) was around 0.17. Each culture was grown aerobically or anaerobically and then collected at 13 h (late exponential/early stationary phase) or 26 h (late stationary phase) postinoculation as pH was adjusted every 4 h. The experiments were done in triplicate. For anaerobic culture, medium was nitrogen purged and tightly capped in a completely sealed flask. Cell density was measured at 600 nm using a spectrophotometer (SmartSpec Plus; Bio-Rad).

**Measurement of glucose and ethanol concentrations.** Glucose concentrations were measured using the YSI 7100 multiparameter bioanalytical system (YSI Life Sciences, Yellow Springs, OH). Ethanol concentrations were measured using a UV-based ethanol assay kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's protocol.

**Total RNA preparation.** Total RNA was prepared according to a protocol previously published (31) for all the growth conditions tested. Briefly, cells were grown aerobically or anaerobically and collected at 16 h after inoculation for deep sequencing. All centrifugation was performed at 4°C. Cells were pelleted and resuspended in 1 ml of TRIzol reagent (Invitrogen). Following pelleting, cells were transferred to screw-cap tubes containing glass beads (Sigma) and incubated at 25°C for 5 min. Cells were lysed using a mini-beadbeater (Biospec), with 100-s pulses three times. Cells were kept on ice for 10 min between each 100-s treatment. The beads and cellular debris were centrifuged at 4°C for 2 min. The supernatant was transferred to a clean siliconized 2-ml tube. After addition of 300  $\mu\text{l}$  of chloroform-isoamyl alcohol mixture (24:1, vol/vol), the samples were inverted for 15 s and then incubated at 25°C for 3 min. Then, tubes were centrifuged at 13,000 rpm for 10 min, and the aqueous top phase was transferred to a clean siliconized 1.5-ml tube. Following this step, 270  $\mu\text{l}$  of isopropanol and 270  $\mu\text{l}$  of a mixture of 0.8 M sodium citrate and 1.2 M sodium chloride were added. The samples were mixed well and then incubated on ice for 10 min. The RNA was pelleted by centrifugation at 13,000 rpm for 15 min. The pellet was washed with 1 ml of 95% cold ethanol and centrifuged for 5 min. The pelleted RNA was allowed to air dry for ~5 min and was resuspended in 30  $\mu\text{l}$  of RNase-free water (Ambion). The RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (Thermo). Samples were stored at -20°C. Total RNAs were validated on 10% urea gels to verify the quality of the RNAs and make sure that the RNAs did not undergo any degradation.

**Deep sequencing for RNA and data processing.** Prepared RNA was quantified and qualified using a bioanalyzer before sequencing. The NEB-Next Small RNA Library Prep Set for Illumina (New England BioLabs Inc.) was used for generating small RNA libraries. Sequencing was performed using an Illumina HiSeq 2000 with paired-end 100-base reads (Genomic Sequencing and Analysis Facility at the University of Texas at Austin). Prior to analyzing sequencing results, the adapter sequences were trimmed to remove low-quality bases at the ends of the reads. Data were processed using BWA (Burrows Wheeler Aligner) (32) and mapped onto the *Z. mobilis* ZM4 complete genome (GenBank accession number

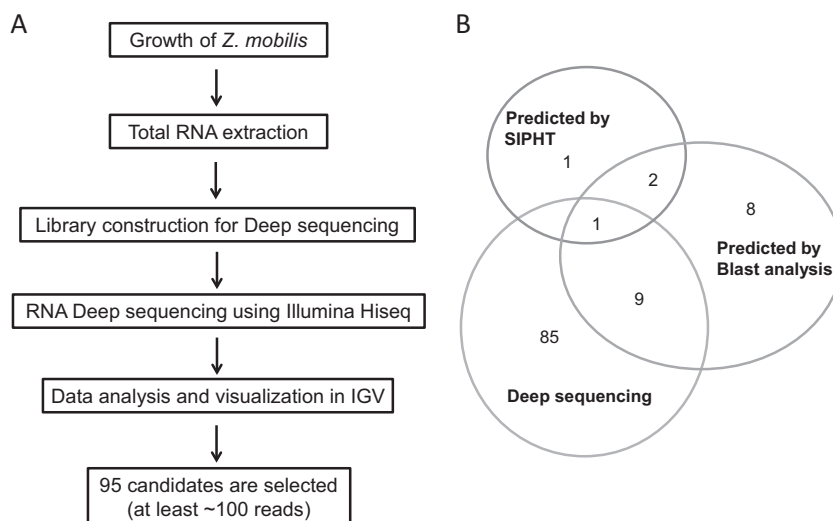
NC\_006526). The mapped sequencing reads were visualized in Integrated Genome Viewer (IGV) (33).

**Computational analysis of predicted sRNA by Blast.** Sequence conservation analysis of intergenic regions was implemented using WU-BLAST (blastn 2.0MP-WashU [4 May 2006]; W. Gish, personal communication). WU-BLAST output was filtered with a PERL script to a stringent threshold of at least 50% query sequence coverage with 50% identity in the conserved region. These parameters were selected according to search criteria that have been developed to analyze the conservation levels of protein-encoding sequences, where the expected level of conservation is much higher. We categorized within a genus and outside a genus for the data analysis.

**Northern blotting.** Small RNA Northern blotting was performed as described in reference 31. Briefly, Northern blotting analysis was performed to verify expression of potential sRNAs candidates that resulted from computational predictions and transcriptomic analysis. DNA oligonucleotide probes specific for each candidate sRNA (see Table S1 in the supplemental material) were labeled using 20 pmol of oligonucleotide in a 20- $\mu\text{l}$  kinase reaction mixture containing 25  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP and 20 U of T4 polynucleotide kinase (NEB) at 37°C for 1 h. A ladder ( $\Phi\text{X174}$  DNA/HinfI [Promega]) was labeled in the same manner. Total RNA (50  $\mu\text{g}$  to ~100  $\mu\text{g}$ ) was separated on a 10% denaturing polyacrylamide gel that was then transferred to a positively charged membrane (Hybond N+; GE Life Sciences) for blotting. Hybridization was performed using Amersham Rapid-hyb buffer (GE Healthcare) by following their recommended protocol for oligonucleotide probes, with a 3-h incubation or overnight incubation at 42°C. After three washing steps with washing buffer (5 $\times$  SSC-0.1% SDS [1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate] for the 1st washing and 1 $\times$  SSC-0.1% SDS for the 2nd and 3rd washing steps), membranes were exposed to a phosphor screen overnight and visualized with a phosphorimager (Typhoon imager; Amersham Biosciences).

**Deep 5' and 3' RACE.** Deep rapid amplification of cDNA ends (RACE) was performed using total RNA samples from both aerobic and anaerobic cultures. 5' deep RACE was performed using the Ion Torrent 316 chip (Wadsworth Center Applied Genomic Technologies Core Facility) as previously described (23, 34). Briefly, the FirstChoice RLM-RACE kit (Ambion) was used with minor modifications to the protocol. A total of 8  $\mu\text{g}$  of RNA was treated with tobacco acid pyrophosphatase (TAP) at 37°C for 1 h, followed by ligation of the 5' RACE kit adapter at 37°C for 1 h. The resulting RNA was then reverse transcribed according to the manufacturer's protocol. PCR was performed on the resulting cDNA. All primer sequences used for deep RACE are listed in Table S2 in the supplemental material. To increase the yield of some sRNAs, PCRs were reamplified using the product from the original reaction as a template and the same primers. Resulting PCR products were purified using the QIAquick PCR purification kit (Qiagen) and RNase-free water (Ambion) for final elution. All products were pooled.

For 3' RACE, a published protocol (34) was followed, using a miScript reverse transcription kit (Qiagen) to perform reverse transcription. PCR was performed on the resulting cDNA. Resulting PCR products were purified using a QIAquick PCR purification kit (Qiagen) and eluted in RNase-free water (Ambion). Sequences of all primers used were listed (see Table S2 in the supplemental material). All products were pooled and sequenced using an Ion Torrent 316 chip at the Wadsworth Center Applied Genomic Technologies Core Facility. Data analysis was done using public resources in the Galaxy website (<http://usegalaxy.org/>). To analyze the sequencing results for the 5' and 3' RACE, adapter sequences were first removed for each sample and then sequences lacking 5' or 3' adapter sequences were removed. After analysis of the sequencing results, data were mapped onto the *Z. mobilis* ZM4 complete genome sequence (NC\_006526) using Bowtie2 (35) with default parameters and visualized with IGV (33).



**FIG 1** Experimental scheme for sRNA candidate selection. (A) This schematic shows the strategy for selecting sRNA candidates from deep-sequencing methods. (B) Each number of candidate sRNAs from experimental and computational approaches is shown in the Venn diagram.

**Nucleotide sequence accession number.** Newly determined sequencing data reported in this manuscript are available from Gene Expression Omnibus (GEO) under accession number [GSE57773](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57773).

## RESULTS
















**Transcriptome analysis of *Zymomonas mobilis* for identifying putative small RNAs.** A combination of computational and experimental methods was used in this work to identify novel sRNAs in *Z. mobilis*. First, we isolated total RNA from cells cultured under anaerobic conditions (as higher growth rates are observed under these conditions for *Z. mobilis*) and conducted a high-throughput transcriptome sequencing analysis using Illumina Hiseq. Prior to sequencing, RNA quantification and quality assessment were performed with a bioanalyzer. Following mapping of sequencing results to the *Z. mobilis* complete genome (NCBI reference sequence [NC\\_006526.2](https://www.ncbi.nlm.nih.gov/nuccore/NC_006526.2)), all hits were visualized using Integrative Genomics Viewer (IGV; <http://www.broadinstitute.org/igv/>). The experimental search scheme is outlined in [Fig. 1A](#). Importantly, we identified a total of 95 candidates that mostly represented highly expressed transcripts (having over 100 mapped sequence reads; at least 10% of the average number of reads observed in tRNAs). Although we expected that slightly expressed sRNA candidates could also have an important role in regulation (36), our initial focus on highly expressed candidates stemmed from our interest in further confirming expression of these sRNAs via Northern blotting and in fully mapping the transcript ends. These sRNA characterization techniques are known to be more robust with larger sRNA quantities (37). It is worthwhile to note that in this study, we narrowed our search to intergenic sRNA candidates. Our rationale for excluding sequences that even partially overlapped known open reading frames is that intergenic candidates have a lower possibility of representing fragmented mRNA transcripts or other degradation products.

**Computationally predicted sRNAs in *Zymomonas mobilis*.** As a complementary technique to sRNA identification in *Z. mobilis*, we used a combination of computational approaches that have proven successful in our previous work (23). Our interest in complementing our experimental search with such approaches

stemmed from the fact that even though RNA sequencing is a powerful transcriptome analysis technique, it can only capture transcripts expressed during the particular experimental condition under which cells are collected for RNA preparation. It is therefore not surprising that computational predictions have also become widely used for the discovery of small regulatory RNAs in bacteria (14, 21). We performed two specific computational prediction approaches to identify novel sRNA candidates in *Z. mobilis*: (i) SIPHT (sRNA identification protocol using high-throughput technologies) (38) and (ii) a bioinformatics analysis recently developed in our laboratory (unpublished data) based on the search of long and conserved intergenic regions. Using SIPHT, we identified 4 novel sRNA candidates. As a side note, SIPHT predicts intergenic loci in any of the over 1,500 bacterial replicons in the NCBI database guided by sequence conservation upstream of putative Rho-independent terminators (38).

In addition to using SIPHT to identify potential sRNA targets, we performed a genome-wide BLAST conservation and size analysis of all 1,011 intergenic regions that have not been annotated to be gene encoding in *Z. mobilis* and predicted 20 additional candidates (see Materials and Methods for a detailed description). These predictions take advantage of sRNA enrichment trends that we have previously established in long and highly conserved regions of multiple bacterial genomes (we plan to publish the results in the future). Results from all bioinformatics studies are listed in Table S3 in the supplemental material. Collectively, 106 sRNA candidates were identified from computational analysis and experimental approaches (see Table S4 in the supplemental material).

When comparing results from our computational analysis, we found that 3 out of 4 candidates predicted by SIPHT were also identified in our bioinformatics analysis. However, only 10 of the 85 candidates that were selected from the analysis of deep sequencing data were also predicted computationally. The combined experimental and computational scheme for selecting sRNA candidates is summarized in [Fig. 1A](#). [Figure 1B](#) shows the overlap in sRNA predictions from all methods used in this work.

		5' end	3'end	New nomenclature	Size (nt)	Prediction method	Category
Zms1		1080003	1079429	ncZMO11069Ac	1300	Computation (BLAST, SIPHT) Experiment	overlap with adjacent gene
Zms2		<b>1223382</b>	<b>1223136</b>	ncZMO1198Ac	72-118	Computation (BLAST)	overlap with adjacent gene
Zms3		512975	513362	ncZMO10513A	234-271	Computation (BLAST)	intergenic
Zms4		<b>1351044</b>	1350765	ncZMO11136Ac	271	Experiment	intergenic
Zms6		<b>454669</b>	<b>454972</b>	ncZMO10460A	271-310	Experiment	intergenic
Zms8		<b>157766</b>	<b>157687</b>	ncZMO0173Ac	200	Experiment	overlap with adjacent gene
Zms9		<b>1564777</b>	<b>1564963</b>	ncZMO115135A	118	Experiment	intergenic
Zms10		<b>39274</b>	<b>39493/39703</b>	ncZMO0037A	603/310	Experiment	overlap with adjacent gene
Zms13		540051	540556	ncZMO10546Ac	500	Computation (BLAST) Experiment	intergenic
Zms14		<b>1242778</b>	1242990	ncZMO11218A	~72/194-234	Computation (BLAST, SIPHT)	intergenic
Zms15		<b>1666899</b>	1666996	ncZMO11624A	550/1300	Computation (BLAST) Experiment	intergenic
Zms16		<b>868928</b>	<b>869052</b>	ncZMO10860A	72-118	Experiment	intergenic
Zms18		<b>1901203</b>	1900964	ncZMOr005Ac	310	Experiment	overlap with adjacent gene
Zms20		<b>258449</b>	<b>258569</b>	ncZMO10256A	115	Experiment	intergenic
Zms24		<b>1607606</b>	<b>1607830</b>	ncZMO11574A	194-234	Experiment	intergenic

**FIG 2** Summary of validated sRNA candidates. Properties of Zms1 to Zms24 are shown. The approximate sRNA size observed by Northern blotting corresponds with 5' and 3' deep RACE results. Coordinates in bold were verified with 5' and 3' deep RACE. Other coordinates are from predicted coordinates from a computational search or calculated from Northern blotting. Arrows between coding genes are represented sRNAs, and the direction of arrows shows the orientation of each sRNA. All prediction methods are shown. Identified sRNAs are classified into two categories: entirely intergenic or overlapping adjacent genes.

Strikingly, one only candidate was identified by all prediction methods; this further highlights the different pools of potential sRNA candidates that were tapped into by these methods.

**High-throughput validation of sRNAs using Northern blotting.** To validate sRNA expression from the pool of all candidates, we performed large-scale Northern blotting. Cells were grown anaerobically and collected for RNA extraction in stationary phase given that *Z. mobilis* Hfq has been shown to be more abundantly expressed in anaerobic, stationary phase than

in aerobic, stationary phase (26). As Hfq is known as a global sRNA regulator (39), we reasoned that there was a higher chance to identify (and experimentally validate) sRNAs under this condition. A list of all the probes used for Northern blotting is included in Table S1 in the supplemental material. Given that deep-sequencing data did not provide strand information, sRNAs were probed on both the plus strand and the minus strand. In addition, each candidate was probed with at least two different probes. Importantly, expression of a total of 15 can-

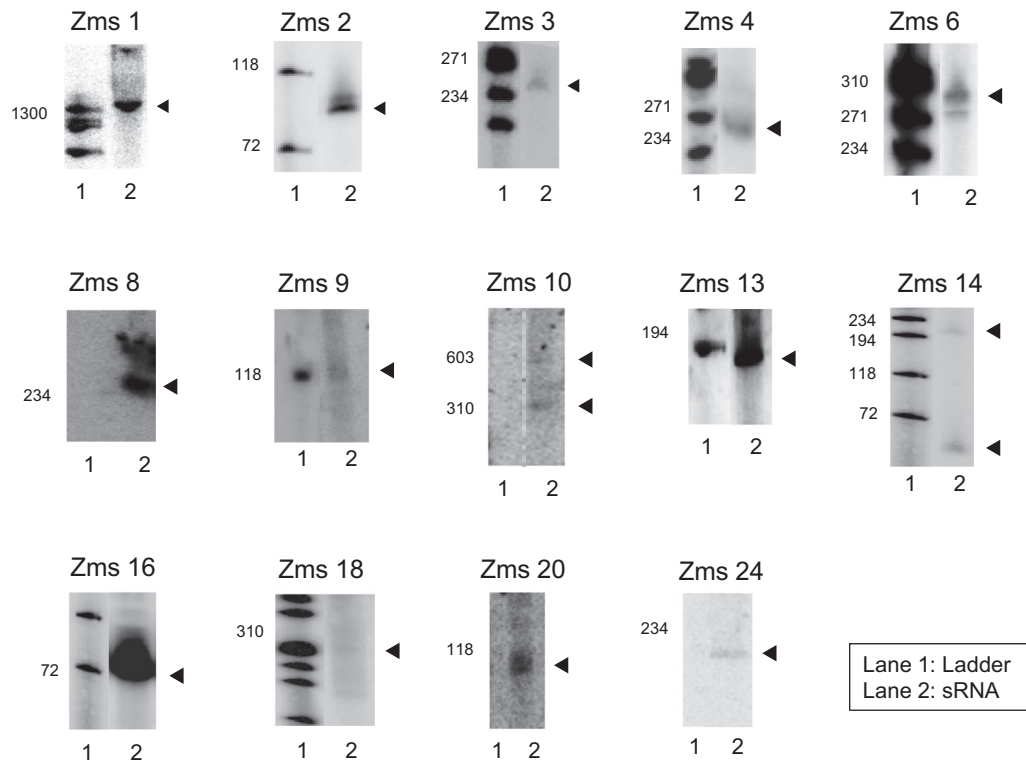


FIG 3 Representative Northern blots for confirmed sRNA in *Z. mobilis*. Northern blotting was performed to examine the expression of candidate sRNAs. Representative blots were confirmed with at least two different probes (see Table S1 in the supplemental material). A black arrowhead indicates the sRNA band for each candidate. Lane 1,  $\Phi$ X174 DNA-HindIII-digested ladder; lane 2, sRNA.

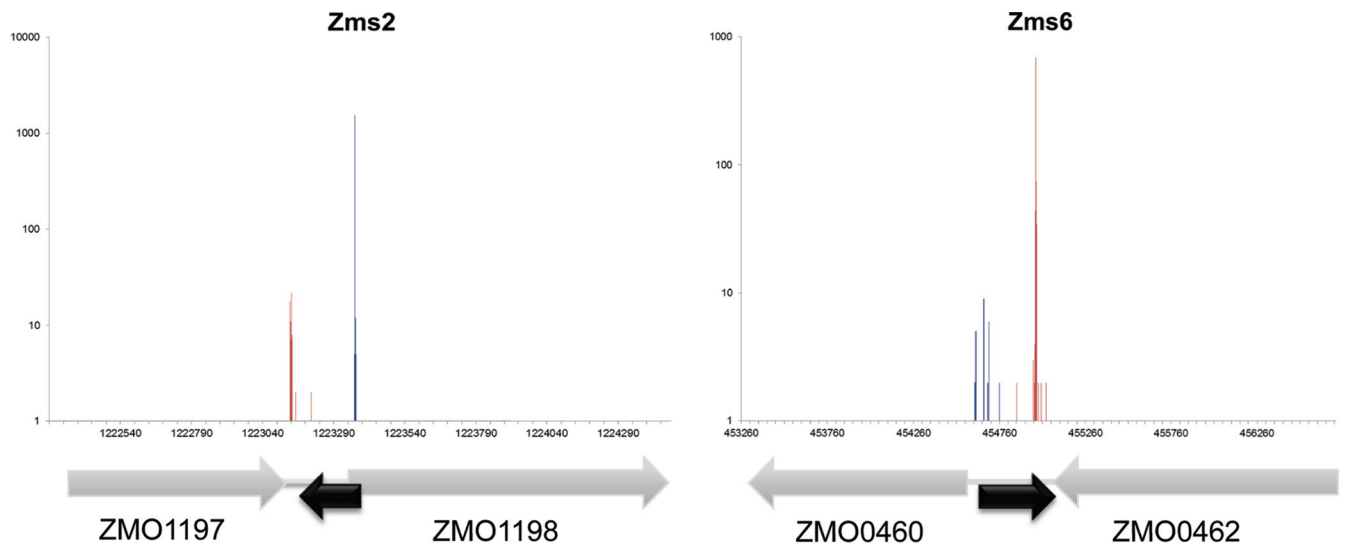
didates was confirmed with multiple probes, designed to bind different regions of the putative sRNA transcript.

Figures 2 and 3 show a summary of all the confirmed sRNAs as well as an image of the positive signal obtained by Northern blotting using their corresponding probes. Confirmed sRNAs were originally enumerated with a designated Zms (*Zymomonas mobilis* sRNA) nomenclature, but they were then annotated according to a published system for bacterial sRNAs (40). As indicated in Fig. 2, 12 of the confirmed sRNAs were identified from the high-throughput sequencing analysis and 3 were identified computationally; 3 sRNAs were found from both prediction methods. It is worthwhile pointing out the presence of multiple bands in some of our samples; these could represent degradation products or several transcription products from the same region. Importantly, the same patterns were observed despite the use of different probes for each region.

**Mapping of transcription start and end site by 5' and 3' deep RACE.** Given that high-throughput transcriptome analysis does not provide precise information of transcriptional start and end sites, we adapted deep 5' and 3' RACE analysis for precise mapping of transcript ends and for further confirmation of sequencing results. This method combines conventional RACE technique with deep-sequencing technology for the efficient verification of transcription start and end site in sRNA candidates (34, 41). Coordinates for the 5' and 3' ends of each sRNA from deep RACE analysis are shown in Fig. 2. Figure 4 shows representative data for mapping transcription start and end sites from 5' and 3' deep RACE for 2 selected sRNA candidates. As determined by comparing the lengths of confirmed sRNAs, the results are in agreement

with previous results confirmed by Northern blotting. Mapping results of all sRNAs by deep RACE with adjacent genes are shown in Fig. S1 in the supplemental material.

**Differential expression of sRNA candidates under different conditions of ethanol production.** To understand how expression of the confirmed sRNAs could change under different conditions of ethanol accumulation, we cultured cells under anaerobic and aerobic conditions. We pursued the analysis of all confirmed sRNAs under both aerobic and anaerobic conditions, as these could be important to basic cellular functions and to the regulation of ethanol production and/or tolerance, respectively. It has been known that the lack of oxygen positively affects glucose consumption, ethanol accumulation, and growth in *Z. mobilis* (4). To achieve conditions that show differential production of ethanol, *Z. mobilis* was grown aerobically and anaerobically. As shown in Fig. 5A, the maximal growth rates of *Z. mobilis* under aerobic and anaerobic conditions (estimated as  $0.26 \text{ h}^{-1}$  and  $0.28 \text{ h}^{-1}$ , respectively) did not show a significant difference. In addition, we verified established trends in glucose consumption and ethanol production under these conditions. After 26 h of culture, 84.23 mM and 169.74 mM ethanol were measured under aerobic and anaerobic conditions, respectively. As shown in Fig. 5B, glucose is consumed faster under the anaerobic conditions and the corresponding production of ethanol is more rapid under anaerobic conditions. These trends were also consistent with previously published reports (4) and confirmed that the desired culturing conditions were achieved. After screening all confirmed sRNAs, one of the most interesting aspects of this work was the finding that 3 sRNAs (Zms2, Zms4, and Zms6) showed differential expression under aerobic and anaerobic culture conditions (Fig. 5C). Zms2 and Zms6 showed 0.8-



**FIG 4** Representative 5' and 3' deep RACE data. Two representative (Zms2 and Zms6) deep RACE graphs are shown. Blue lines show the number of 5' RACE reads mapped to respective genome, while red lines show the number of 3' RACE reads. The black arrow under the chart shows where the sRNA is located, and the gray arrows represented the adjacent annotated coding regions.

fold and 0.64-fold decreases, respectively, in expression level under anaerobic culture condition. Inversely, Zms4 showed 1.5-fold-increased expression under anaerobic culture conditions. These results suggested the possibility that these sRNAs could be functionally associated with the metabolic regulation of ethanol production and/or ethanol tolerance.

**Differential expression of sRNA candidates is responsive to environmental growth conditions.** After confirming differentially expressed sRNAs under different levels of ethanol, we next tested directly the effect of ethanol stress on the expression of all identified sRNAs. Previous work had shown that coordinated changes in expression of specific heat shock proteins and metabolic enzymes (e.g., alcohol dehydrogenase) are important under high ethanol stress (42, 43). This supported the possibility that sRNAs could also be differentially expressed as potential posttranscriptional regulators under high ethanol stress conditions. Therefore, we systematically tested expression levels of all identified sRNA candidates under the ethanol stress conditions. We chose a 5% (vol/vol) ethanol supplement to the medium as the ethanol stress conditions given that 6% (vol/vol) ethanol was previously shown to affect cell viability dramatically (44). We confirmed that Zms2, Zms6, and Zms18 showed differential expression under ethanol stress conditions (Fig. 6A). Interestingly, Zms2 and Zms6 also exhibited differential expression between aerobic and anaerobic conditions. In contrast, Zms18 only showed differential expression between 0% ethanol-supplemented growth conditions and 5% ethanol-supplemented growth conditions, indicating its potential involvement in the regulation of the ethanol tolerance in *Z. mobilis*. In the case of Zms4, even though it was observed to be expressed at higher levels under anaerobic conditions (relative to aerobic conditions), it was not observed to be differentially expressed between 0% and 5% ethanol stress conditions (data not shown). A plausible possibility is that Zms4 is more involved in managing oxygen stress.

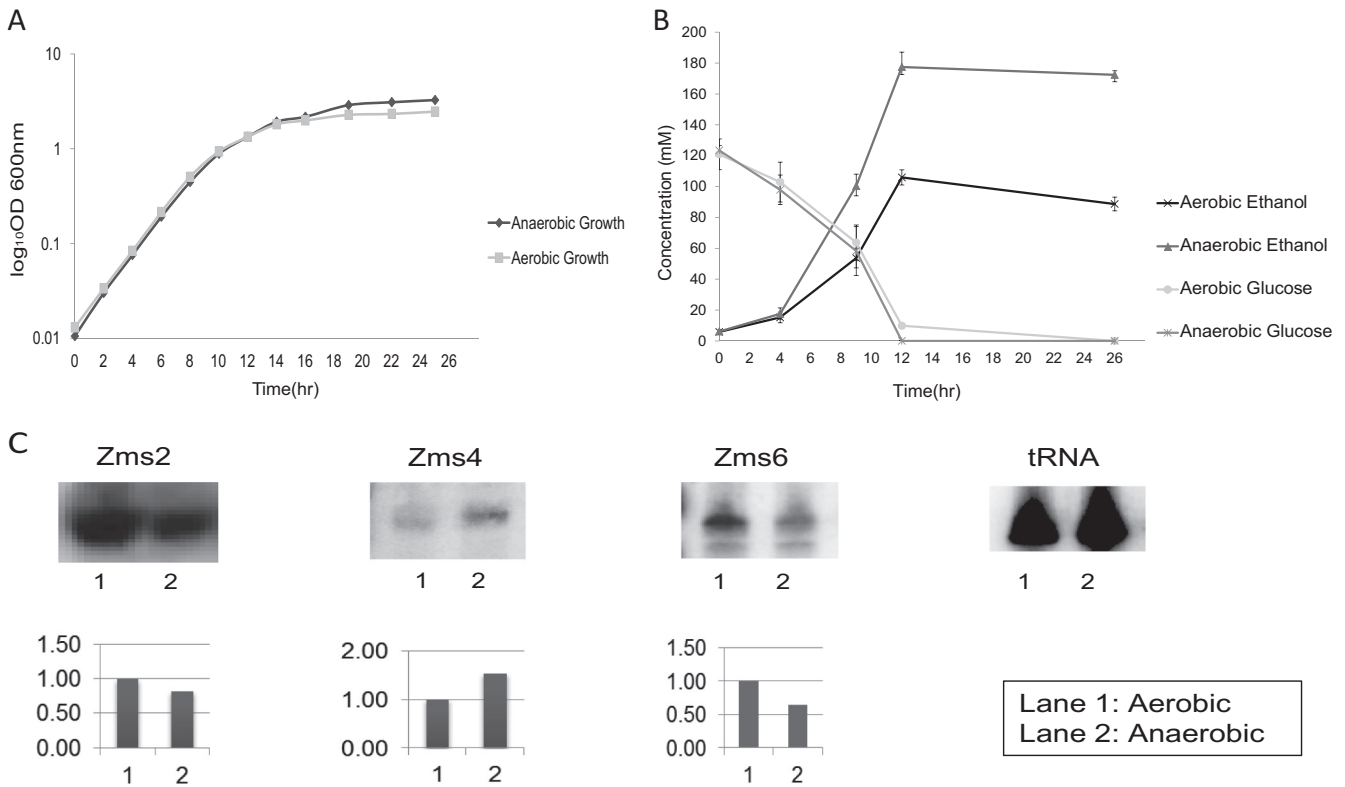
Lastly, all experiments described above were done under conditions of late exponential phase. Given variations in gene expres-

sion levels that have been confirmed under different growth phases in *Yersinia* and *Mycobacterium* (45, 46), we reasoned that functional sRNAs could also be differentially expressed under different growth phases in *Zymomonas*. Interestingly, Hfq, which is known as an RNA chaperone in bacteria (25, 47), has been identified in *Z. mobilis* and showed greater expression in anaerobic stationary phase (4). To test for differential sRNA expression as a result of different growth phases, we harvested total RNA samples from cells collected at 13 h postinoculation (late exponential phase) and 26 h postinoculation (late stationary phase). Importantly, Zms2 and Zms6 also showed differential expression between early and late stationary phase (Fig. 6B). Both sRNAs accumulate until late exponential phase and then decrease in late stationary phase.

**Target prediction of verified sRNAs.** As the underlying mechanism of sRNAs depends on base-pairing, many computational resources for sRNA target prediction are available. Given the importance of identifying sRNA targets for unraveling their underlying mechanistic roles, we performed an initial target prediction analysis using TargetRNA2 (<http://cs.wellesley.edu/~btjaden/TargetRNA2/>) as a preliminary study. We focused on Zms2, Zms4, Zms6, and Zms18, which were differentially expressed under different stress conditions. Table 1 shows the list of target genes obtained using TargetRNA2. All listed targets have a *P* value of <0.001. In case of Zms2, there is an ABC (ATP-binding cassette) transporter gene in the list. Recently, an ABC transporter gene has been found as a target gene for sRNAs in *Clostridium*, and it could be related to antibiotic resistance (27); however, we blasted the sequence of this sRNA and that of Zms2, and there is no sequence homology (data not shown). Further analysis will focus on validating these predictions.

## DISCUSSION

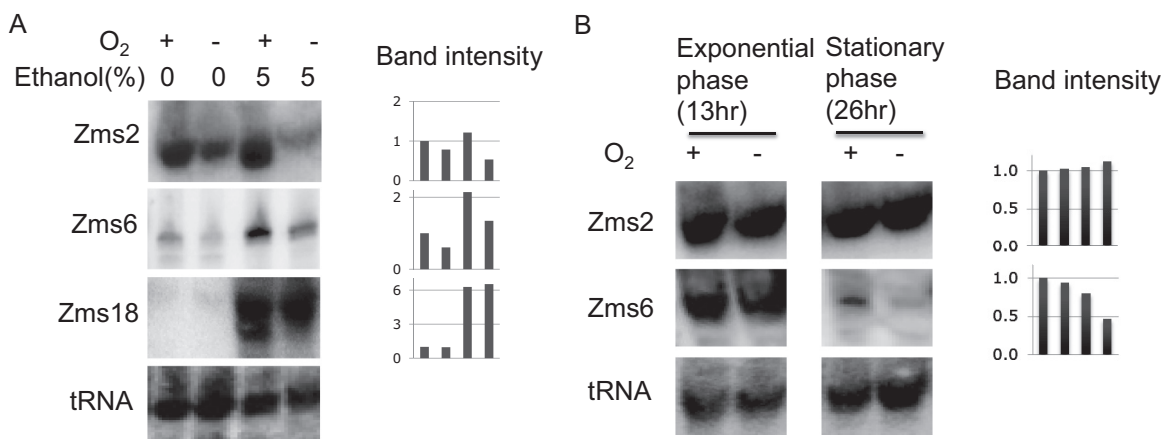
Recent research on *Z. mobilis* has unraveled changes in its transcriptomic and metabolic pathways associated with ethanol metabolism. In this study, we successfully discovered 15 novel sRNAs



**FIG 5** Growth of *Z. mobilis* and differential expression of sRNAs under anaerobic and aerobic conditions. (A) Growth curves under each condition are shown. Mean values  $\pm$  standard deviations (bars) for triplicates are shown for each condition. The OD<sub>600</sub> of cells was measured every 2 h. (B) Ethanol production and glucose consumption are shown. Mean values  $\pm$  standard deviations (bars) for triplicates are shown for each condition. (C) The expression of sRNA for each candidate is shown with the corresponding intensity of each band detected by Northern blotting. Zms2 (~90 bp), Zms4 (~271 bp), and Zms6 (~290 bp) showed differential expression between aerobic and anaerobic conditions. Band intensities were normalized based on those of tRNA.

in *Z. mobilis* utilizing experimental and computational approaches. Although 106 candidates selected from our combinatorial methods were tested by Northern blotting, expression was confirmed for only 15 sRNAs. It is worth noting the possibility that many of the candidates, identified by transcriptomic or com-

putational analysis, were below the detectable threshold by Northern blotting under the experimental conditions used in this study. Compared to mapped reads of tRNAs in deep sequencing, which were mapped with an average of 1,500 reads, identified sRNA candidates showed very low read numbers. This could partially



**FIG 6** Expression patterns of sRNAs under ethanol stress and various growth conditions. (A) Addition of ethanol affects the expression levels of sRNAs. The first two lanes show results from medium with no ethanol supplementation, and last two lanes show sRNA expression under 5% ethanol supplementation. All samples were collected under 13 h (late exponential phase) after inoculation. Aerobic and anaerobic conditions are shown as O<sub>2</sub> + and -, respectively. Band intensities were normalized based on tRNA expression levels. (B) Different growth phase samples were collected 13 h (late exponential phase) or 26 h (late stationary phase) after inoculation for Northern blotting. Band intensities were normalized based on tRNA expression levels.

TABLE 1 Genes for sRNAs from target analysis

sRNA	Target prediction result ( $P < 0.001$ )
Zms2	ABC transporter (ZMO0175) Outer membrane autotransporter barrel domain-containing protein (ZMO0213) Hydroxylamine reductase (ZMO0117) Citrate synthase (ZMO1963) TonB-dependent receptor (ZMO0031)
Zms4	Glutaredoxin-like protein (ZMO1873) CheX protein (ZMO0084)
Zms6	Chemotaxis protein CheD (ZMO0080) Nitrogen fixation protein (ZMO1829) 7-Cyano-7-deazaguanine reductase (ZMO0326) Ppx/GppA phosphatase (ZMO0403)
Zms18	Cation efflux protein (ZMO0204)

explain why we detected only 14% of sRNAs by Northern blotting even though we used an excessive amount of total RNA (up to 100  $\mu$ g) for detection. Despite high levels of total RNA used for testing, the intensity of some detected sRNAs is very low; this attests to the limitation of Northern blotting as an experimental tool for sRNA validation. Furthermore, total RNA, used for deep sequencing and Northern blotting, was extracted from cells only under limited physiological conditions. This limited number of growth conditions can also explain why some predicted sRNAs were not detected experimentally, since they could still be transcribed under different conditions that we have not tested.

Besides the analysis of sRNA candidates from deep-sequencing data, we also analyzed expression of mRNAs under aerobic and anaerobic conditions. When we compared our results with published transcriptomic analysis data (4), we found that most upregulated and downregulated genes under aerobic conditions were also identified in our study (see Table S5 in the supplemental material). We confirmed that Entner-Doudoroff pathway genes were more abundant under anaerobic conditions. We also observed that several transcription and response regulators are upregulated in aerobic conditions. Additionally, we detected about 200 upregulated genes and 62 downregulated genes under aerobic conditions. Most of the newly found genes in our study are related to metabolism and cellular processes. Our analysis showed that alcohol dehydrogenase, which was downregulated under ethanol treatment conditions (44), was less abundant in late exponential phase under aerobic conditions (see Table S5). We also confirmed that the Hfq gene (ZMO0347) was more abundant under anaerobic conditions. Differences in our data relative to previously published microarray data (4) (particularly in the fold changes detected) could be explained by the increase sensitivity of deep-sequencing methods and by the collection of samples under different growth phases.

It is also worthwhile to point out that although we initially selected sRNA candidates from the intergenic regions, upon confirmation by Northern blotting, some sRNAs were detected to be longer than predicted. Our 5' and 3' deep RACE results further confirmed that some sRNAs overlapped with 5' or 3' ends of adjacent genes. Thus, we categorized our identified sRNAs into two groups based on their location: intergenic sRNAs and overlapping sRNAs. Intergenic sRNAs are transcribed from intergenic

regions between adjacent genes. On the other hand, overlapping sRNAs can be located at the 5' untranslated regions (UTRs) of adjacent genes to function as riboswitches (48) or can also be generated from mRNA posttranslational processing if encoded from the 3' end of the adjacent gene. It has been known that sRNAs can be transcribed from independent promoters or derived from processing of mRNA UTRs (49, 50). There are several pieces of evidence that the sRNAs identified in this study are not fragments of mRNAs: (i) many sRNAs are transcribed in different orientations from adjacent genes, and (ii) our several Northern blots showed no larger bands that could correspond to preprocessed mRNAs. Even though it is unlikely that any of the sRNAs we identified in this study were generated by mRNA processing, it is well established that regulatory sRNAs can be derived from processing of mRNA UTRs (51, 52).

To further characterize the uncovered sRNAs, we confirmed their expression levels under ethanol levels (5%) that have been reported to stress cell growth and decrease ethanol productivity (53). Three sRNAs (Zms2, Zms6, and Zms18) were expressed differentially under ethanol stress, suggesting that they could be related to regulatory mechanisms of ethanol production or tolerance in *Z. mobilis*. Analysis of comprehensive comparison with transcriptomic and proteomic data under this condition might be the next step for defining targets of sRNAs to understand regulatory mechanisms. Likewise, we uncovered in our studies two sRNAs (Zms2 and Zms6) that accumulate until late exponential phase and then decrease in late stationary phase. In *Yersinia*, some sRNAs showed the same pattern of expression under late exponential and stationary phase, and these differential levels of sRNA expression correlated with Hfq expression (45). Therefore, we speculate that function of these sRNAs might be Hfq dependent. Further analysis should be performed to understand the role of Hfq in *Zymomonas mobilis*. Lastly, it is noteworthy that preliminary target prediction analysis shows ABC transporter genes as a putative target of Zms2. However, there is no conservation in sequence of Zms2 in other organisms. Ongoing studies are focusing on elucidating the metabolic roles of Zms2 and other differentially expressed sRNAs under ethanol. As part of these future efforts, we are focusing on experimental validation of the targets identified by computational prediction methods.

This study reinforces the importance of sRNA-associated mechanisms for engineering of microbes that are relevant to the production of biofuels. Interestingly, sRNA regulation could also be exploited in the metabolic synchronization of ethanologenic organisms within consortia. This strategy is already being explored to increase levels of ethanol production involving cocultures of bacteria and yeast (54).

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