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ARTICLE

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Intracellular accumulation of c-di-GMP and its regulation on self-flocculation of the bacterial cells of *Zymomonas mobilis*

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Abstract

Zymomonas mobilis is an emerging chassis for being engineered to produce bulk products due to its unique glycolysis through the Entner–Doudoroff pathway with less ATP produced for lower biomass accumulation and higher product yield. When self-flocculated, the bacterial cells are more productive, since they can self-immobilize within bioreactors for high density, and are more tolerant to stresses for higher product titers, but this morphology needs to be controlled properly to avoid internal mass transfer limitation associated with their strong self-flocculation. Herewith we explored the regulation of cyclic diguanosine monophosphate (c-di-GMP) on self-flocculation of the bacterial cells through activating cellulose biosynthesis. While ZMO1365 and ZMO0919 with GGDEF domains for diguanylate cyclase activity catalyze c-di-GMP biosynthesis, ZMO1487 with an EAL domain for phosphodiesterase activity catalyzes c-di-GMP degradation, but ZMO1055 and ZMO0401 contain the dual domains with phosphodiesterase activity predominated. Since c-di-GMP is synthesized from GTP, the intracellular accumulation of this signal molecule through deactivating phosphodiesterase activity is preferred for activating cellulose biosynthesis to flocculate the bacterial cells, because such a strategy exerts less perturbance on intracellular processes regulated by GTP. These discoveries are significant for not only engineering unicellular *Z. mobilis* strains with the self-flocculating morphology to boost production but also understanding mechanism underlying c-di-GMP biosynthesis and degradation in the bacterium.

KEYWORDS
c-di-GMP, chassis, industrial production, microbial cell factories, self-flocculation, *Zymomonas mobilis*

1 | INTRODUCTION

Zymomonas mobilis is a Gram-negative and facultatively anaerobic bacterium, which produces ethanol through the Entner–Doudoroff (ED) pathway (Kalnenieks, 2006). Compared with the Embden–Meyerhof–Parnas (EMP) pathway that is commonly employed by other microorganisms, the ED pathway produces less ATP for lower biomass accumulation, since ATP is dissipated predominantly through biosynthesis, in particular cell growth (Xia et al., 2019). From the viewpoint of mass balance, more sugar can be directed to ethanol production with observed yield improved, which is very important for producing ethanol as a biofuel with major cost from sugar consumption (Gombert & van Maris, 2015).

On the other hand, the bacterial cells are smaller than the brewing yeast *Saccharomyces cerevisiae* for a high specific surface to assimilate sugar faster, which, together with the low energy-coupling ED pathway, forms a catabolic highway for carbon metabolism to produce ethanol quickly (Rutkis et al., 2016). Moreover, *Z. mobilis* can

be engineered with pentose metabolism through the isomerase pathway without cofactor imbalance for intermediate accumulation (Zhang et al., 1995), an intrinsic drawback for engineering *S. cerevisiae* with the redox pathway for the same purpose (Endalur Gopinarayanan & Nair, 2019).

These merits make *Z. mobilis* suitable for being engineered to produce not only cellulosic ethanol, but also other bulk products from lignocellulosic biomass (He et al., 2014). ZM401, a mutant developed from ZM4, the unicellular model strain of *Z. mobilis*, can self-flocculate with advantages for industrial production (Cao et al., 2022). When self-flocculated, the bacterium can self-immobilize within bioreactors conveniently for high cell density to improve productivity without extra cost as that occurs with conventional cell immobilization using supporting materials, which was highlighted previously in ethanol fermentation with the self-flocculating yeast (Zhao & Bai, 2009). In addition, the bacterial flocs can be recovered through cost-effective gravity sedimentation instead of centrifugation, a regular practice for harvesting unicellular cells with high capital investment on centrifuges and intensive energy consumption on running these facilities.

Tolerance to environmental stresses is a prerequisite for microbial strains to be robust in production, since various stresses are present under industrial production conditions (Gong et al., 2017). Product inhibition is common, because high product titers have been pursued endlessly in industry to save energy consumption on product recovery, and in the meantime reduce wastewater discharge, which has been highlighted in high-gravity ethanol fermentation (Puligundla et al., 2019). Toxicity from by-products is one of the biggest challenges for lignocellulose bioconversion to produce biofuels and bio-based chemicals, since various toxic byproducts including furfural, 5-hydroxymethylfurfural, and acetic acid are generated inevitably during the pretreatment of lignocellulosic biomass (Ling et al., 2014).

Although various technologies, such as physical adsorption, chemical treatment, and biological degradation, have been developed for detoxifying lignocellulosic hydrolysate, none of them is economically feasible for industrial applications (Nogueira et al., 2021). Meanwhile, tolerance to individual stress such as ethanol, acetic acid, and high temperature has been studied for *Z. mobilis* (Carreón-Rodríguez et al., 2019; Li et al., 2021; Yang et al., 2020), but the progress is less significant, because multiple stresses are always co-existed under industrial production conditions, and thus general stress response is preferred (Guan et al., 2017). Self-flocculation of the bacterial cells of *Z. mobilis* improves their tolerance to elevated ethanol and inhibitors presented in lignocellulosic hydrolysate as well (Zhao et al., 2014).

Chemical basis for self-flocculation of the bacterial cells of ZM401 was experimentally validated to be cellulose fibrils (Xia et al., 2018), which are synthesized in the mutant more efficiently by the bacterial cellulose synthase (Bcs) complex due to single nucleotide polymorphism (SNP) mutations occurred on the genes ZMO1082 and ZMO1055 (Cao et al., 2022). As a second messenger, cyclic diguanosine monophosphate (c-di-GMP) regulates intracellular processes through a dynamic balance between the biosynthesis and degradation of this signal molecule, which are catalyzed by diguanylate cyclase (DGC) and phosphodiesterase (PDE), respectively (Jenal et al., 2012; Ute Römling & Amikam, 2006). Since bacterial cellulose biosynthesis is regulated by c-di-GMP (Morgan et al., 2014; Ross et al., 1987), we reason that the intracellular accumulation of c-di-GMP in *Z. mobilis* could impact self-flocculation of the bacterial cells through its regulation on the biosynthesis of cellulose fibrils.

In this work, we explored genes encoding enzymes related to c-di-GMP metabolism in *Z. mobilis*, identified new genes with functions on the biosynthesis and degradation of this signal molecule, and studied its intracellular accumulation for impact on self-flocculation of the bacterial cells. The progress is significant for not only engineering unicellular strains from *Z. mobilis* with such a multicellular morphology for robust production, but also understanding mechanism underlying c-di-GMP metabolism through its intracellular biosynthesis and degradation in the bacterium.

2 | MATERIALS AND METHODS

2.1 | Strains, media, and culture

All strains used in this work are listed in Supporting Information: Table S1. *Z. mobilis* stocks were inoculated into the rich medium (RM) composed of 10 g/L yeast extract, 20 g/L glucose, and 2 g/L KH_2PO_4, and grown statically at 30°C until exponential phase, which were then transferred into 250 mL flasks, each containing 100 mL RM medium with 10% inoculation for subculture to increase OD_{600} to ~1.0. In case of need, 20 μg/mL tetracycline was supplemented into the RM medium. Congo red staining was used to visualize cellulose produced by *Z. mobilis*, and 2 μL of the subculture was inoculated onto the RM agar plate containing 70 μg/mL Congo red, which was incubated for 12–24 h at 30°C for visual inspection (Thongsomboon et al., 2020; Trivedi et al., 2016).

2.2 | Development of recombinant strains

Primers and plasmids used in this work are listed in Supporting Information: Tables S2 and S3. To construct gene knockout mutants, the suicide vector pEX18Tc was used (Hoang et al., 1998). Fragments of 500–1000 bp for flanking genes to be deleted were amplified from *Z. mobilis*, which were cloned into *Escherichia coli* DH5α for amplification. Then, recovered plasmids were fused with enzymatically digested pEX18Tc, and confirmed by sequencing. Subsequently, the plasmids were transformed into *E. coli* JM110 for demethylation to be transformed into *Z. mobilis* ZM4 or ZM401 more efficiently through electroporation. The RM medium supplemented with tetracycline was used to select colonies harboring the target plasmids, and 5% sucrose was supplemented into the RM medium for the counter-selection of the homologous colonies (Li et al., 2013). The selected mutants were verified through tetracycline sensitivity

and Colony PCR. The shuttle vector pHW20a was used to carry target genes (Dong et al., 2011). Genes to be overexpressed were colonized from *Z. mobilis*, which, together with the promoter of glyceraldehyde-3-phosphate dehydrogenase gene (Pgap), were amplified by PCR for fusion with pHW20a. The expression plasmids were demethylated in *E. coli* JM110, and then transformed into *Z. mobilis* as previously described (Xia et al., 2018). When engineered with the empty vector pHW20a, no significant difference was observed compared to their wild-type strains ZM4 and ZM401, respectively (Supporting Information: Figure S1). Therefore, ZM4/pHW20a and ZM401/pHW20a were used as the control for comparative analysis. All engineered strains were verified by PCR.

2.3 | Characterization for self-flocculation of *Z. mobilis*

The culture of 4 mL was sampled, and de-flocculated by cellulases following the protocol established previously for measuring OD_{600} using the Microplate Reader (Thermo Fisher) (Xia et al., 2018), which was used as the basis (A) for quantifying the flocculation efficiency of *Z. mobilis*. The culture collected simultaneously was rested statically for 5 min, and then 400 µL supernatant was sampled, which was also treated with cellulases to de-flocculate any suspended small flocs, and mixed vigorously for homogeneous suspension to measure OD_{600} (*B*). The flocculation efficiency (*F*) of *Z. mobilis* was calculated using the equation:

$$F = (1 - B/A) \times 100\%.$$

2.4 | Extraction and quantification of intracellular c-di-GMP

c-di-GMP was extracted as reported previously with modifications (Xu et al., 2013). Briefly, 15 mL culture of *Z. mobilis* grown to exponential stage was centrifuged at 5000*g* for 3 min. The pellet was immediately suspended in 1000 µL buffer (40% methanol:40% acetonitrile:20% dH_2O) by vigorous vortex at −20°C for 30 min, which was then centrifuged at 12,000*g* and 4°C for 5 min, and the supernatant was collected into a tube cooled with ice. The cell debris was extracted twice, and the supernatant was collected. All supernatant was combined for vacuum evaporation to condense c-di-GMP, which was dissolved into 80 µL buffer (50% acetonitrile and 50% dH_2O) for analysis.

c-di-GMP quantification was performed using the Waters I-Class Acquity UPLC coupled with the Vion IMS QToF mass spectrometer (Waters). The separation of c-di-GMP was performed using the SeQuant ZIC-HILIC column (100 mm × 2.1 mm) packed with 3.5 µm polyetheretherketone and operated at 45°C, and the mobile phase composed of 50 mM ammonium formate in water (A) and acetonitrile (B) was pumped at 0.4 mL/min under the following gradient elution conditions: 0–10 min, 90–50% B; 10–12 min, 50–90% B; 12–15 min, 90% B. c-di-GMP was detected through electrospray ionization operated at the negative-ion mode. The software UNIFI 1.8.1 was used for data processing.

c-di-GMP with a purity of 98% (Biolog) was used as the standard to calibrate the analysis.

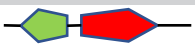
3 | RESULTS

3.1 | Role of c-di-GMP in self-flocculation of the bacterial cells

In the genome of ZM4, ZMO1055, ZMO0401, ZMO1487, ZMO1365, and ZMO0919 are predicted to encode proteins with conserved domains of GGDEF and/or EAL for DGC and/or PDE activity, catalyzing the biosynthesis and degradation of c-di-GMP, respectively (Jones-Burrage et al., 2019). While ZMO1055 and ZMO0401 contain both GGDEF and EAL domains, ZMO1487 contains an EAL domain only, and both ZMO1365 and ZMO0919 contains GGDEF domains (Table 1).

To explore the role of the intracellular accumulation of c-di-GMP in self-flocculation of *Z. mobilis*, genes *wspR* and *yhjH* encoding DGC and PDE in *Pseudomonas aeruginosa* and *Escherichia coli* for c-di-GMP

TABLE 1 Protein domains predicted for c-di-GMP metabolism in *Z. mobilis* (ZM4).

Protein ID	Domain for c-di-GMP metabolism	Domain architecture	ORF (aa)
ZMO1055	GGDEF/EAL with activity of cyclase/phosphodiesterase for c-di-GMP synthesis/degradation		579
ZMO0401	GGDEF/EAL with activity of cyclase/phosphodiesterase for c-di-GMP synthesis/degradation		683
ZMO1487	EAL with activity of phosphodiesterase for c-di-GMP degradation		258
ZMO0919	GGDEF with activity of cyclase for c-di-GMP synthesis		1008
ZMO1365	GGDEF with activity of cyclase for c-di-GMP synthesis		1045

Abbreviations: aa, amino acid; ORF, open reading frame.

beta-propeller signal transduction domain; periplasmic ligand-binding sensor domain; GGDEF domain for cyclase activity; EAL domain for phosphodiesterase activity; domain for transmembrane helixes.

biosynthesis and degradation (Güvener & Harwood, 2007; Lindenberg et al., 2013) were overexpressed in ZM4 (ZM4/*wspR*) and ZM401 (ZM401/*yhjH*), respectively. These manipulations would enhance or compromise the intracellular accumulation of c-di-GMP within the bacterial cells.

While ZM4/*wspR* self-flocculated incompletely for a loose and flake-like morphology (Figure 1a) with flocculating efficiency of 30.9% compared to that of 6.1% observed in the control ZM4/pHW20a, the self-flocculating phenotype of the control ZM401/pHW20a was disrupted completely with flocculating efficiency decreased to 5.5% (Figure 1b). On the other hand, the intracellular accumulation of c-di-GMP increased drastically to 72.7 pg/mg protein in ZM4/*wspR* from 10.0 pg/mg proteins detected for the control, but only 1.4 pg (c-di-GMP)/mg protein was detected in ZM401/*yhjH*, compared to that of 14.3 pg/mg protein detected in the control (Figure 1b).

Previous studies confirmed that cellulose fibrils are the chemical basis for developing the self-flocculating phenotype in ZM401 (Xia et al., 2018). Congo red binds to 1,4-β-D-glucopyranosyl units with strong affinity, which can be used to characterize cellulose production qualitatively (Trivedi et al., 2016). When cultures were inoculated onto agar plates containing the rich medium (RM) supplemented with Congo red and incubated properly, dark red was observed for ZM401/pHW20a due to more cellulose production, but weak staining reaction occurred with ZM4/pHW20a, ZM4/*wspR*, and ZM401/*yhjH*, indicating that less cellulose was produced in ZM4, even when it was engineered with the overexpression of *wspR* for synthesizing more c-di-GMP, and also in ZM401 when it was engineered with the overexpression of *yhjH* for c-di-GMP degradation (Figure 1c). Therefore, intracellular accumulation of c-di-GMP regulates the development of the self-flocculating phenotype in *Z. mobilis*, but synthesis of sufficient cellulose by the bacterial cells would be a prerequisite for them to self-flocculate with significance for industrial applications (Cao et al., 2022).

3.2 | Endogenous c-di-GMP metabolism in *Z. mobilis*

Comparative genome analysis between ZM401 and ZM4 detected the SNP mutation in ZMO1055: thymine replaced by cytosine for the amino acid substitution Ala526Val, and its role in the degradation of c-di-GMP and development of the self-flocculating phenotype in ZM401 was studied recently (Cao et al., 2022). However, less attention has been paid on the role of the wild-type ZMO1055 ($ZMO1055^+$) from ZM4 in the biosynthesis and degradation of c-di-GMP.

When $ZMO1055^+$ was overexpressed in ZM401, its self-flocculating phenotype was disrupted with the flocculating efficiency decreased to 5.0%, and the intracellular accumulation of c-di-GMP decreased drastically to 0.44 pg/mg protein, compared to that of 92.5% and 14.25 pg/mg protein, respectively, observed in the control ZMO401/pHW20a (Figure 2). However when ZMO1055 with the SNP mutation from ZM401 ($ZMO1055^-$) was overexpressed in ZM401, its self-flocculating phenotype was compromised slightly with the flocculating efficiency decreased to 85.9% only, and the intracellular accumulation of c-di-GMP compromised less to 6.07 pg/mg protein, indicating that the SNP mutation substantially compromised the PDE activity of $ZMO1055^+$ for c-di-GMP degradation, which was supported by the deletion of $ZMO1055^-$ from ZM401 for the signal molecule to further increase to 16.68 pg/mg protein (Figure 2). Manipulations of $ZMO1055^+$ and $ZMO1055^-$ in ZM4 through their overexpression and deletion also indicated a strong PDE activity of the wild-type protein for c-di-GMP degradation.

To further confirm the PDE activity, we constructed an expression plasmid carrying $ZMO1055^+$ but with the site-directed mutation for the amino acid substitution Ala356Glu to change the catalytic domain from EAL to AAL, which was experimentally validated to deactivate PDE activity in *P. aeruginosa* (Kuchma et al., 2007; Nesbitt et al., 2015). When $ZMO1055^-$ was knocked

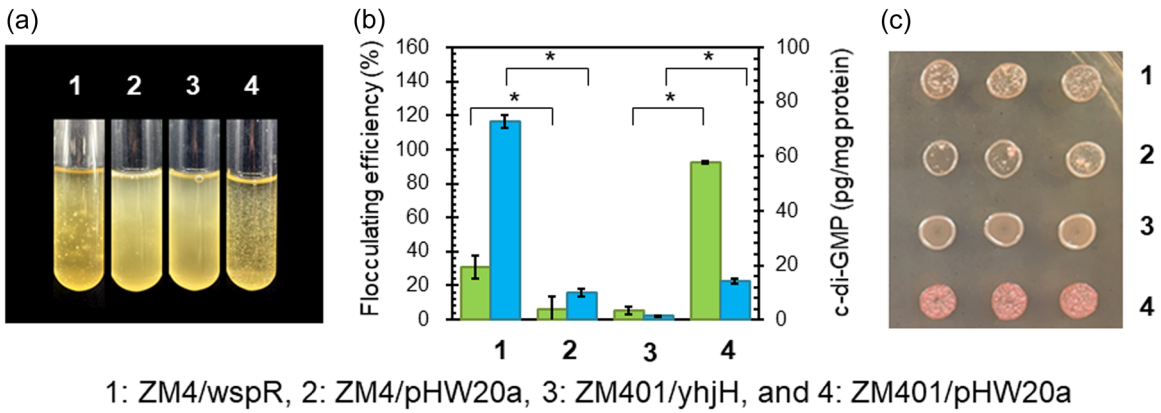


FIGURE 1 Overexpression of *wspR* encoding diguanylate cyclase from *P. aeruginosa* in ZM4 (ZM4/wspR) and *yhjH* encoding phosphodiesterase from *E. coli* in ZM401 (ZM401/yhjH). (a) Phenotypes observed on the engineered strains cultured in the rich medium. (b) Flocculating efficiency (■) and intracellular accumulation of c-di-GMP (■) of the bacterial cells, and (c) qualitative characterization of extracellular cellulose by Congo red staining. ZM4/pHW20 and ZM401/pHW20: ZM4 and ZM401 engineered with the empty vector pHW20a as the control. Error bars represent standard deviation for triplicates, and the significance was analyzed by the *t*-test with $^*p < 0.05$.

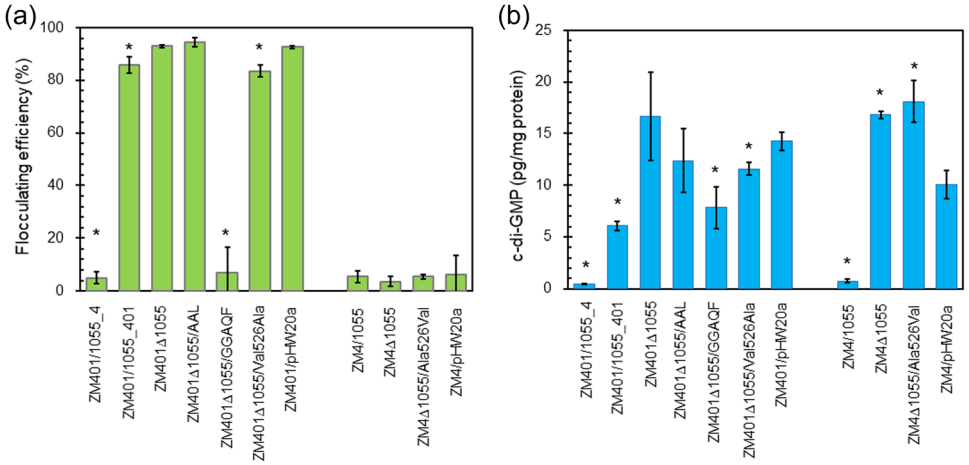


FIGURE 2 Manipulations of ZMO1055 and impact on self-flocculation of the bacterial cells of *Z. mobilis* (a), and intracellular accumulation of c-di-GMP (b). (+) and (−): wild type and mutated genes from ZM4 and ZM401. (/) and (Δ): gene overexpression and deletion. ZM401ΔZMO1055$^-$/1055$^+$(AAL) and ZM401ΔZMO1055$^-$/1055$^+$(GGAQF): ZM401 manipulated with the deletion of ZMO1055$^-$ and overexpression of the recombinant plasmid bearing the domain AAL or GGAQF developed through site-directed mutations in ZMO1055$^+$ for the amino acid substitute Ala356Glu and Asp232Ala, respectively. ZM401ΔZMO1055$^-$/1055$^-$ (V526A): ZM401 engineered with the deletion of ZMO1055$^-$ and overexpression of the recombinant plasmid bearing ZMO1055$^-$ with the site-directed reverse mutation for the amino acid substitute Val526Ala. ZM4ΔZMO1055$^+$/1055$^+$(A526V): ZM4 engineered with the deletion of ZMO1055$^+$ and overexpression of the recombinant plasmid bearing ZMO1055$^+$ with the site-directed mutation for the amino acid substitute Ala526Val. ZM4/pHW20 and ZM401/pHW20: ZM4 and ZM401 engineered with the empty plasmid pHW20 as the controls. Error bars represent standard deviation for triplicates, and the significance was analyzed by the *t*-test with $^*p < 0.05$ compared to that detected in ZM4/pHW20a or ZM401/pHW20a, respectively.

out, and the recombinant plasmid was transformed into ZM401, no significant change was observed on the intracellular accumulation of c-di-GMP, and the mutant ZM401ΔZMO1055$^-$/1055^{AAL} still maintained the self-flocculating phenotype (Figure 2). These experimental results indicate that the substitution Ala526Val on ZMO1055$^+$ occurred in ZM401 did compromise the protein's PDE activity for c-di-GMP degradation as the native gene bearing the AAL domain exhibited. Such a conclusion was validated by the reverse substitution Val526Ala in ZM401 (ZM401ΔZMO1055$^-$/1055$^+$), as well as the substitution of Ala526Val in ZM4 (ZM4ΔZMO1055$^+$/1055$^-$) to compromise the protein's PDE activity for intracellular accumulation of c-di-GMP to as high as 18.1 pg/mg protein (Figure 2).

ZMO1055 also contains GGDEF (GGDQF) domain to catalyze the biosynthesis of c-di-GMP. When GGDQF was replaced by GGAQF through the substitution of Asp232Ala to deactivate the protein's DGC activity in ZM401, the intracellular accumulation of c-di-GMP decreased to 7.82 pg/mg protein, about 50% of that detected in the control, and its self-flocculating phenotype was disrupted completely (Figure 2). These results indicate the DGC activity of ZMO1055, and also its contribution to the biosynthesis of c-di-GMP for the intracellular accumulation of this signal molecule as well as the development of the self-flocculating phenotype in ZM401.

ZMO0401 is another protein with the dual domains speculated for the biosynthesis and degradation of c-di-GMP in *Z. mobilis*. When it was overexpressed, ZM401 lost the self-flocculating phenotype with the flocculating efficiency decreased to 5.6%, and in the meantime extremely low intracellular accumulation of c-di-GMP

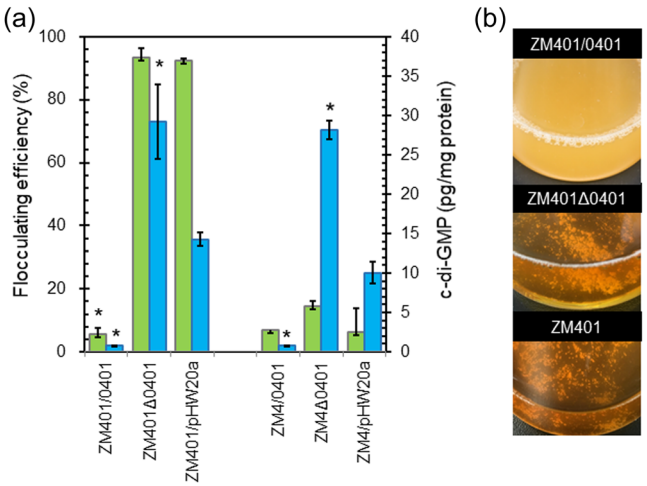


FIGURE 3 Contribution of ZMO0401 to self-flocculation of the bacterial cells of *Z. mobilis* (■) and intracellular accumulation of c-di-GMP (■) (a), and morphologies observed in flask cultures when shaking was stopped for 3−5 s (b). (/) and (Δ): gene overexpression and deletion. ZM4/pHW20 and ZM401/pHW20: ZM4 and ZM401 engineered with the empty vector pHW20a as the controls. Error bars represent standard deviation for triplicates, and the significance was analyzed by the *t*-test with $^*p < 0.05$ compared to that detected in ZM4/pHW20a or ZM401/pHW20a, respectively.

(0.76 pg/mg protein) was detected, indicating that ZMO0401 might function predominantly on c-di-GMP degradation (Figure 3). We therefore constructed the knockout mutants ZM401Δ0401 and ZM4Δ0401, respectively, to validate such a speculation.

When ZMO0401 was deleted from ZM401, the intracellular accumulation of c-di-GMP increased to 29.2 from 15.5 pg/mg protein, but no significant change in the self-flocculating phenotype was observed for the mutant, indicating that the intracellular accumulation of c-di-GMP was high enough for activating the biosynthesis of cellulose to flocculate the bacterial cells. As for ZM4, the intracellular accumulation of c-di-GMP increased significantly from 9.8 to 28.2 pg/mg protein when ZMO0401 was deleted, but the flocculating efficiency of ZM4Δ0401 increased slightly to 15.5%, due to its inability to synthesize sufficient cellulose, which we previously showed relies on a mutation in the protein ZMO1082 involved in cellulose biosynthesis (Cao et al., 2022) (Figure 3).

ZMO1487 was predicted to encode a protein with an EAL domain only for c-di-GMP degradation. When ZMO1487 was overexpressed in ZM401 and ZM4, respectively, the intracellular accumulation of c-di-GMP decreased drastically to 0.95 and 0.54 pg/mg protein from 15.48 to 10.04 pg/mg protein detected in the controls, and the self-flocculating phenotype of ZM401 was disrupted (Figure 4). On the other hand, when ZMO1487 was deleted from ZM4, intracellular accumulation of c-di-GMP increased to 17.62 pg/mg protein, but no significant difference was observed when ZMO1487 was deleted from ZM401 (Figure 4). The reason for this phenomenon might be due to relatively weak impact of ZMO1487 on c-di-GMP metabolism in ZM401 compared to ZMO1055 and ZMO0401, particularly when the SNP mutation in ZMO1055 substantially compromised its PDE activity and enhanced the intracellular accumulation of c-di-GMP. These experiments indicate the catalytic function of the EAL domain in ZMO1487 on c-di-GMP degradation.

Both ZMO1365 and ZMO0919 were predicted to encode DGC domains only for c-di-GMP biosynthesis. Compared to the intracellular accumulation of c-di-GMP at 9.8 pg/mg protein in the control, the overexpression of ZMO1365 and ZMO0919 in ZM4 increased its intracellular accumulation of c-di-GMP to 82.49 and 27.77 pg/mg protein, respectively (Figure 4b). The extremely high intracellular accumulation of the signal molecule stimulated partial development of the self-flocculating phenotype in the mutants with their flocculating efficiency increased to 46.1% and 30.1%, compared to only 5.5% observed in the control (Figure 4b). These experimental results validated the catalytic function of the DGC domain in ZMO1365 and ZMO0919 on c-di-GMP biosynthesis.

3.3 | Engineering ZM4 with the self-flocculating phenotype

With elucidation on functions of genes involved in the biosynthesis and degradation of c-di-GMP, we targeted genes encoding proteins with PDE activity for c-di-GMP degradation to explore the effect of their combinatory knockout on developing the self-flocculating phenotype in ZM4.

When both ZMO1055 and ZMO0401 were deleted, the flocculating efficiency of the bacterial cells was improved to 24.4%, and the flocculating efficiency was further improved to 34.5% for the double knockout mutant with both ZMO1055 and ZMO1487 deleted (Figure 5a). No further improvement on the self-flocculating phenotype was observed when all the three genes were deleted from ZM4. However, such a genetic manipulation would be preferred for reducing the genome of *Z. mobilis* to engineer this species as a more reliable chassis to accommodate heterogeneous genes more effectively.

Cellulose fibrils were validated to be the chemical basis for developing the self-flocculating phenotype in ZM401 (Xia et al., 2018). ZM4 also contains a bacterial cellulose synthase (*bcs*) operon composed of ZMO1082, ZMO1083, ZMO1084, and ZMO1085. ZMO1082 was predicted as a putative gene encoding a short peptide composed of 67 amino acid residues only (Xia et al., 2018), which is less likely to be functional, and thus can be manipulated together with ZMO1083. We therefore engineered ZM4 with the overexpression of ZMO1082-1083, ZMO1082-1084, and the whole *bcs*

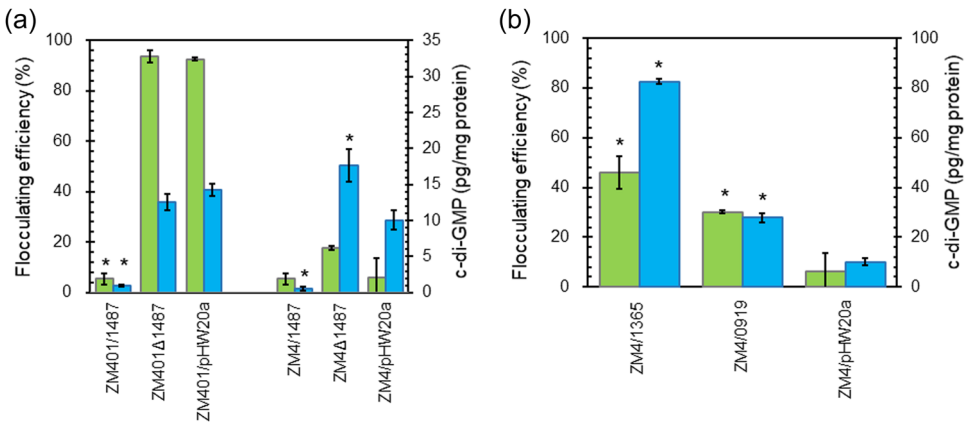


FIGURE 4 Impact of the deletion (Δ) and overexpression (/) of ZMO1487 (a), and ZMO1365 and ZMO0919 (b) on the self-flocculation (■) of the bacterial cells of *Z. mobilis* and intracellular accumulation of c-di-GMP (■). ZM4/pHW20 and ZM401/pHW20: ZM4 and ZM401 engineered with the empty vector pHW20a as the controls. Error bars represent standard deviation for triplicates, and the significance was analyzed by the *t*-test with $^*p < 0.05$ compared to that detected in ZM4/pHW20a or ZM401/pHW20a, respectively.

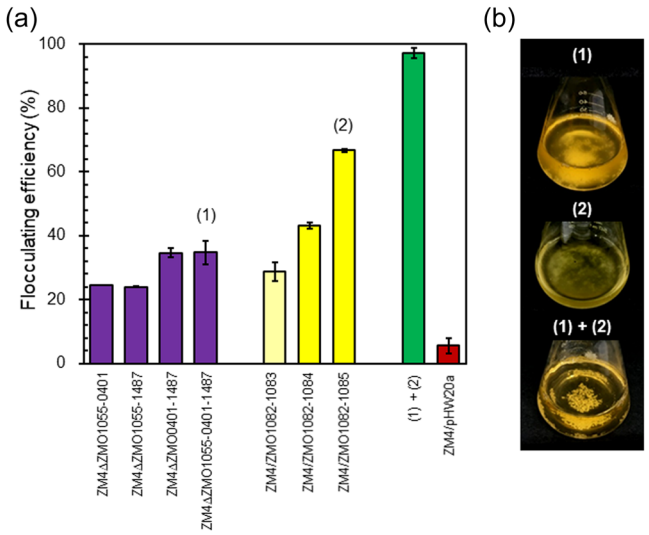


FIGURE 5 Engineering ZM4 (a) with the deletion (Δ) of ZMO1055, ZMO0401 and ZMO1487, combinationally (■), overexpression (/) of ZMO1082-1083, ZMO1082-1084, and ZMO1082-1085 (■) as well as the deletion of ZMO1055, ZMO0401, and ZMO1487 (1), together with the overexpression of the whole *bcs* operon ZMO1082-1085 (2), for strategy (1) + (2) to develop the self-flocculating phenotype (■), and the morphologies observed in flask culture of the engineered strains (b). ZM4/pHW20: ZM4 engineered with the empty vector pHW20a as the control (■). Error bars represent standard deviation of triplicates.

operon ZMO1082-1085, respectively, to investigate their contribution to the self-flocculation of the bacterial cells. As a result, the development of the self-flocculating phenotype was observed in the mutants with their flocculating efficiency of 28.9%, 43.1%, and 66.7%, respectively (Figure 5a).

When both the two strategies were employed in ZM4: enhancing its intracellular accumulation of c-di-GMP to 94.42 pg/mg protein through the deletion of ZMO1055, ZMO0401, and ZMO1487, and the biosynthesis of cellulose fibrils through the overexpression of the *bcs* operon, the flocculating efficiency of 97.3% was observed for the bacterial cells, which is higher than that of 92.5% detected with ZM401 (Figure 5a). Morphologies are further shown for ZM4 strains engineered with the deletion of ZMO1055, ZMO0401, and ZMO1487, the overexpression of the *bcs* operon, and the combination of these two strategies (Figure 5b).

When industrial strains are engineered with new phenotypes, such as the self-flocculation of microbial cells for more advantages, their production performance should not be compromised. *Z. mobilis* is ethanologenic, and suitable for producing cellulosic ethanol. Therefore, we compared ethanol fermentation performance between the strain engineered with the self-flocculating phenotype and its unicellular wild-type ZM4. As can be seen (Supporting Information: Figure S2), no difference was observed when medium supplemented with 100 g/L glucose, equivalent to total sugars in the hydrolysate of lignocellulosic biomass, was fermented to produce ethanol.

4 | DISCUSSION

Z. mobilis has been acknowledged as a potential chassis to be engineered for biorefinery of lignocellulosic biomass to produce bulk products with major cost from feedstock consumption such as ethanol as a biofuel and 2,3-butanediol as a building block (Xia et al., 2019; Yang et al., 2016). However, unlike *E. coli* and *S. cerevisiae* that have been intensively studied and delicately engineered as chassis cells (Mienda & Dräger, 2021; Mitsui & Yamada, 2021), much less is known for *Z. mobilis* to be engineered as microbial cell factories for robust production of bulk products, in particular through rational design.

As a signal molecule, c-di-GMP regulates intracellular processes including cellulose biosynthesis in bacteria (Jenal et al., 2012; Morgan et al., 2014; Ross et al., 1987; Ute Römling & Amikam, 2006). Therefore, understanding the biosynthesis and degradation of c-di-GMP in *Z. mobilis* is fundamental for developing this species as a suitable chassis. On the one hand, this knowledge can contribute directly to developing strategies for controlling the morphological shift from unicellular cells to multicellular flocs with physiological and metabolic merits including stress tolerance, and advantages in bioprocess engineering such as biomass recovery and self-immobilization of the bacterial cells within bioreactors. On the other hand, it will benefit for exploring internal cues related to the replication of genetic materials for division and differentiation to support cell growth, since c-di-GMP can act as a cell cycle oscillator to drive chromosome replication (Lori et al., 2015).

Bacteria have evolved with specialized sensory and regulatory domains for responding to c-di-GMP that is accumulated intracellularly at different levels through a dynamic balance between its biosynthesis and degradation, and variants of enzymes with GGDEF or/and EAL domain(s) for DGC or/and PDE activity can fulfill such a task (Hengge, 2021; Petchiappan et al., 2020). ZMO1055, ZMO0401, ZMO1487, ZMO1365, and ZMO0919 are involved with c-di-GMP metabolism in ZM4 for intracellular accumulation of c-di-GMP at different levels, but previously studies confirmed that only ZMO0919 exhibits the DGC activity (Jones-Burrage et al., 2019).

Our experimental results confirmed the function of ZMO0901 as reported before (Jones-Burrage et al., 2019), and in the meantime further validated that all other four genes are functional for c-di-GMP metabolism in ZM4. In addition to ZMO1055 that was studied recently (Cao et al., 2022), the catalytic functions of ZMO0401, ZMO1487, and ZMO1365 on c-di-GMP metabolism in *Z. mobilis* was revealed for the first time. The reason for such a discrepancy might be due to different culture conditions. While rich medium was employed in our studies, minimal medium was used by Jones-Burrage et al. (2019), which could affect the expression of genes and functions of encoded proteins. As a result, these genes could be selected as targets for engineering to explore the role of c-di-GMP in metabolic regulation with *Z. mobilis*.

ZMO1365 and ZMO0919 enhance the biosynthesis of c-di-GMP. Under the catalysis of DGC, c-di-GMP is synthesized from 2 moles of guanosine triphosphate (GTP) with 2 moles of diphosphate

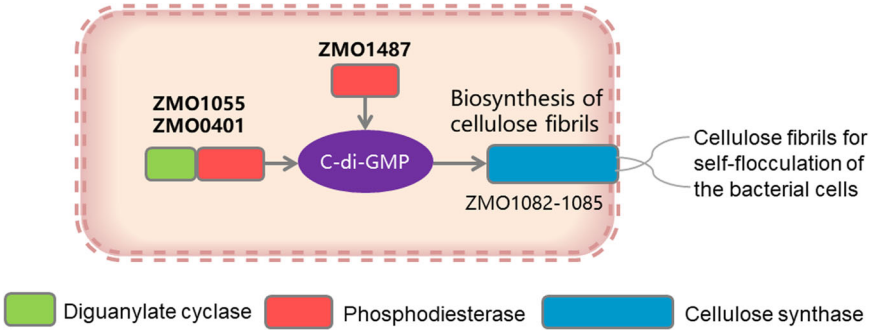


FIGURE 6 Strategies for engineering unicellular *Z. mobilis* strains with the self-flocculating phenotype through enhancing intracellular accumulation of c-di-GMP by the deletion of genes encoding proteins predominantly with the activity of phosphodiesterase to activate cellulose biosynthesis.

produced (Schirmer, 2016). As a high-energy compound, GTP is actively involved in multiple cellular processes such as G-protein signaling through RGS proteins and protein biosynthesis through the GTPase switch regulation (Cherfils & Zeghouf, 2011; Masuho et al., 2020; Wolff et al., 2022), and also acts as a building block for synthesizing RNA during transcription (Akoopie et al., 2020; Attwater et al., 2018). Therefore, energy-intensive GTP production is regulated finely within cells to save the consumption of energy currency in the form of ATP. As a result, the overexpression of ZMO1365 and ZMO0919 in *Z. mobilis* for synthesizing more c-di-GMP from GTP would not be an economic strategy for developing this species as a suitable chassis to be engineered as microbial cell factories, since such a strategy could potentially affect intracellular processes involved with GTP.

It is also interesting that recently ZMO1365 was identified with the mutation of C493T, which not only endowed the *Z. mobilis* strain F211 with tolerance to furfural, but also caused the self-flocculation of the bacterial cells, and the underlying mechanism was attributed to the upregulation of the bacterial cellulose synthase to enhance the biosynthesis of cellulose (Hu et al., 2021). In line with our studies on the function of ZMO1365, we further speculate that the root reason for these phenotypes would be with the impact of such an amino acid mutation on the biosynthesis of c-di-GMP, which consequently activated the bacterial cellulose synthase to synthesize cellulose more efficiently, making the bacterial cells self-flocculated and more tolerant to furfural inhibition as well.

Although ZMO1055 and ZMO0401 encode the dual-functional proteins with both DGC and PDE domains to catalyze the biosynthesis and degradation of c-di-GMP, their PDE activity dominates over DGC activity for c-di-GMP degradation. Moreover, ZMO1487 with PDE activity only catalyzes the degradation of c-di-GMP. Therefore, deactivating PDE activity by deleting ZMO1055, ZMO0401, and ZMO1487 would be preferred for compromising c-di-GMP degradation in *Z. mobilis* to enhance its intracellular accumulation, and consequently activate cellulose biosynthesis to flocculate the bacterial cells (Morgan et al., 2014; Xia et al., 2018). These manipulations would exert less perturbance on other intracellular processes involved with or regulated by GTP.

When engineered only with the overexpression or deletion of genes related to the biosynthesis and degradation of c-di-GMP, ZM4 could not develop the self-flocculating phenotype for applications from the viewpoint of bioprocess engineering. Thus, the overexpression of the whole *bcs* operon composed of ZMO1082-1085 is needed for the bacterial cells to synthesize sufficient amount of cellulose under the regulation of c-di-GMP. Therefore, we propose a strategy for engineering unicellular *Z. mobilis* strains with the self-flocculating phenotype through rational design (Figure 6).

It is worth noting that the size of the bacterial flocs needs to be controlled properly. Large flocs benefit biomass recovery through cost-effective gravity sedimentation, and also could enhance their tolerance to stresses for less demand on detoxification of toxic byproducts in the hydrolysate of lignocellulosic biomass. However, they also present risk for internal mass transfer limitation for substrate transport from bulk environment (outside) into the inner core of the bacterial flocs (inside), and vice versa for transporting product from the inside to outside. No doubt understanding of the regulation of c-di-GMP on self-flocculation of the bacterial cells provides insights on controlling their self-flocculating process at molecule levels, which, together with bioprocess engineering strategies for developing suitable hydrodynamic conditions within bioreactors, could ultimately optimize their size for robust production.

5 | CONCLUSIONS

Intracellular accumulation of c-di-GMP in *Z. mobilis* through its biosynthesis and degradation catalyzed by enzymes with activities of DGC and PDE, respectively, activates the biosynthesis of cellulose fibrils for the bacterial cells to self-flocculate with significant advantages in industrial production. Compared to overexpression of proteins with DGC activity for synthesizing more c-di-GMP from GTP, deactivation of PDE activity to compromise c-di-GMP degradation is more preferred for intracellular accumulation of the signal molecule, since this strategy would exert less perturbance on intracellular processes involved with or regulated by GTP. The progress provides insights into the regulation of c-di-GMP on the morphological shift in *Z. mobilis*, from unicellular cells to multicellular flocs, and also guidelines for engineering unicellular strains from this species and other bacteria with the self-flocculating phenotype through rational design. Moreover, this work lays a basis for exploring mechanism underlying c-di-GMP metabolism in *Z. mobilis* and its regulation on other intracellular processes.

AUTHOR CONTRIBUTIONS

Kai Li drafted the manuscript. Juan Xia performed all experiments and preliminary analysis for the experimental results. Chen-Guang Liu and Feng-Wu Bai co-supervised this work.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data are available in the main text and Supplementary information. Upon request, all materials developed in this work are available for non-commercial use.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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