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

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Abstract

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

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

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Abstract:*Zymomonas mobilis* is an emerging chassis engineered to produce bulk products because of its glycolysis through the Entner-Doudoroff pathway, with less ATP produced for lower biomass accumulation and higher yields with targeted products. When self-flocculated, the bacterial cells are more productive and tolerant to stresses for high product titers, but this morphology needs to be controlled properly to avoid internal mass transfer limitations associated with strong flocculation. In this study, we explored the regulation of cyclic diguanosine monophosphate (c-di-GMP) on self-flocculation of bacterial cells through cellulose biosynthesis. While ZMO1365 and ZMO0919, with GGDEF domains for diguanylate cyclase activities, catalyze c-di-GMP biosynthesis, ZMO1487, with an EAL domain for phosphodiesterase activity, catalyzes c-di-GMP degradation, but ZMO1055 and ZMO0401 contain dual domains with phosphodiesterase activities. Since c-di-GMP is synthesized from GTP, the intracellular accumulation of this signal molecule by deactivating the activity of phosphodiesterase is preferred for activating cellulose biosynthesis to flocculate bacterial cells, since such a strategy exerts less perturbation on intracellular processes regulated by GTP. These discoveries

are significant not only for engineering unicellular *Z. mobilis* strains with a self-flocculating morphology to boost production but also for understanding the mechanism underlying c-di-GMP biosynthesis and degradation in the bacterium.

Keywords: *Zymomonas mobilis* ; chassis; self-flocculation; c-di-GMP; microbial cell factories; industrial production

1 Introduction

Compared with the Embden-Meyerhof-Parnas (EMP) pathway, which is commonly used by other microorganisms, the ethanologenic bacterium *Zymomonas mobilis* employs the Entner-Doudoroff (ED) pathway for glycolysis with less ATP produced for lower biomass accumulation, as ATP is dissipated predominantly through biosynthesis, particularly cell growth of cells (Xia et al., 2019). From the viewpoint of mass balance, more sugar can be directed to ethanol production with improved yield, which is most important for producing ethanol as a biofuel with a major cost from sugar consumption (Gombert et al., 2015). On the other hand, the bacterial cells are smaller than the brewing yeast *Saccharomyces cerevisiae* due to a high specific surface to assimilate sugar faster, which, together with the low energy-coupling ED pathway, forms a catabolic pathway for carbon metabolism to produce ethanol faster (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), which is an intrinsic drawback for engineering *S. cerevisiae* with the redox pathway for the same purpose (Gopinarayanan et al., 2019). These merits make *Z. mobilis* suitable for engineering to produce not only cellulosic ethanol but also other bulk products from lignocellulosic biomass (He et al., 2014).

ZM401, a mutant developed from ZM4, a unicellular model strain of*Z. mobilis* , self-flocculates with advantages for industrial production (Cao et al., 2022). When self-flocculated, bacteria can be conveniently immobilized within bioreactors for high cell density to improve productivity, as highlighted previously in ethanol fermentation with self-flocculating yeast (Zhao et al., 2019). In addition, bacterial flocs can be recovered through cost-effective gravity sedimentation instead of centrifugation, a regular practice for harvesting unicellular cells with high capital investment in centrifuges, as well as intensive energy consumption during their operation, needless to say cost with frequent maintenance.

Tolerance to environmental stresses is a prerequisite for the robust production of microbial strains because various stresses are present under industrial conditions (Gong et al., 2017). Product inhibition is one of these stresses, because a high product titer has been pursued endlessly in industry to save energy consumption on product recovery and reduce the discharge of wastewater, which has been highlighted in high-gravity ethanol fermentation (Puligundla et al., 2019). Toxicity from by-products is another stress, one of the biggest challenges for lignocellulose biorefineries to produce biofuels and bio-based chemicals, as toxic byproducts, including furfural, 5-hydroxymethylfurfural, and acetic acid, are inevitably generated 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 the hydrolysate of lignocellulosic biomass, none of them is economically feasible for industrial applications (Nogueira et al., 2021). Meanwhile, tolerance to individual stresses such as ethanol, acetic acid, and high temperature has been studied for *Z. mobilis*(Carreón-Rodríguez et al., 2019; Yang et al., 2020; Li et al., 2021), but the progress is less significant because multiple stresses always co-exist under industrial production conditions, and general stress responses are preferred (Guan et al., 2017). Self-flocculation with Z. mobilis bacterial cells of *Z. mobilis* can make them more tolerant to elevated ethanol and inhibitors present in the hydrolysate of lignocellulosic biomass (Zhao et al., 2014).

Microbes can develop multicellular morphologies, such as biofilms and activated sludge, under stressful conditions (Ciofu et al., 2022; Wilén et al., 2018). However, self-flocculation with Z. mobilis bacterial cells of *Z. mobilis* presents a unique morphology. Compared to amorphous biofilms, which generally require abiotic surfaces for development with a life cycle (Rumbaugh et al., 2020), no surface is needed for the bacterial cells to self-flocculate, since the process is mediated by cellulose fibers that are self-synthesized (Xia et al., 2018).

Furthermore, a dynamic balance can develop between the breakup of large flocs and the re-flocculation of small flocs under specific hydrodynamic conditions that are developed within bioreactors, which can renew the inside time for the bacterial flocs to sustain viability and perform production efficiently. Unlike activated sludge, which is formed naturally during mixed cultures with abundant microbes as a core community for more efficient syntrophy, such as bacteria for the degradation of short-chain fatty acids and methanogens for methane production in anaerobic digestion (Saunders et al., 2016; Hao et al., 2020), self-flocculation of bacterial cells occurs under pure culture conditions.

The chemical basis for self-flocculation with 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 occurring in 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 its biosynthesis and degradation, which are catalyzed by diguanylate cyclases (DGC) and phosphodiesterases (PDE), respectively (Ute et al., 2006; Jenal et al., 2012). Since bacterial cellulose biosynthesis is regulated by c-di-GMP (Ross et al., 1987; Morgan et al., 2014), we hypothesized that the intracellular accumulation of c-di-GMP in *Z. mobilis* could impact the self-flocculation of the bacterial cells through its regulation of the biosynthesis of cellulose fibrils.

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

2 Materials and Methods

2.1 Strains, media and culture

All strains used in this study are listed in 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, grown statically at 30 °C until their exponential phase, and then transferred into 250 mL flasks, each containing 100 mL RM medium with 10% inoculation for subculture to increase OD_{600} to ˜1.0. When needed, 20 μg/mL tetracycline was added to the RM medium. Congo red staining was used to visualize the cellulose produced by *Z. mobili* s, and 2 μL of the subculture was inoculated onto RM agar plates containing 70 μ μg/mL Congo red, which were incubated for 12-24 h at 30 °C for visual inspection (Trivedi et al., 2016; Thongsomboon et al., 2020).

2.2 Development of recombinant strains

Primers and plasmids used in this study are listed in Tables S2 and S3. 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 *E. coli* DH5α for amplification. The recovered plasmids were fused with enzymatically digested pEX18Tc and confirmed by sequencing. Subsequently, the plasmids were transformed into *E. coli* JM110 for demethylation for efficient transformation into *Z. mobilis* ZM4 or ZM401 through electroporation. RM medium supplemented with tetracycline was used to select colonies harboring the target plasmids, and 5% sucrose was added to the RM medium for counter-selection of homologous colonies (Li et al., 2013). The selected mutants were verified using tetracycline sensitivity and Colony PCR. The shuttle vector pHW20a was used to carry target genes (Dong et al., 2011). The overexpressed genes were colonized from *Z. mobilis* , which, together with the promoter of the 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 with the wild-type strains ZM4 and ZM401 (Fig. S2). Therefore, ZM4/pHW20a and ZM401/pHW20a were used as controls for the comparative analysis. All the engineered strains were verified by PCR.

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

The culture (4 mL) was sampled and de-flocculated by cellulases following a previously established protocol for measuring OD_{600} using a Microplate Reader (Thermo Fisher, Multiskan GO, USA) (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 to obtain a homogenous suspension to measure OD_{600} (B). The flocculation efficiency (F) of *Z. mobilis* was calculated using the following equation:

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

2.4 Extraction and quantification of intracellular c-di-GMP

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

c-Di-GMP quantification was performed using a Waters I-Class Acquity UPLC (Waters, UK) coupled with a Vion IMS QToF mass spectrometer (Waters, UK). The separation of c-di-GMP was performed using a 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 in the negative-ion mode. The software UNIFI 1.8.1 was used for data processing.

c-Di-GMP with a purity of 98% (Biolog, Germany) 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

To explore the role of intracellular accumulation of c-di-GMP in the self-flocculation of *Z. mobilis* , genes *wspR* and*yhjH* encoding DGC and PDE in *Pseudomonas aeruginosa* and*Escherichia coli* for c-di-GMP biosynthesis and degradation (Güvener et al., 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-GMPs in bacterial cells.

While ZM4/*wspR* self-flocculated incompletely for a loose and flake-like morphology (Fig. 1A) with a flocculating efficiency of 30.9% compared to 6.1% observed in the control ZM4/pHW20a, the self-flocculating phenotype of the control ZM401/pHW20a was disrupted completely, and the flocculation efficiency decreased to 5.5% (Fig. 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 (Fig. 1B).

Previous studies have confirmed that cellulose fibrils are the chemical basis for the development of the self-flocculating phenotype in ZM401(Xia et al., 2018). Congo red binds to 1,4-β-D-glucopyranosyl units with a strong affinity, which can be used to qualitatively characterize cellulose production qualitatively (Trivedi et al., 2016). When the 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 cellulose production, but a weak staining reaction occurred with ZM4/*wspR* , ZM4/pHW20a, and ZM401/*yhjH* , indicating that less cellulose was produced (Fig. 1C). Therefore, intracellular accumulation of c-di-GMP regulates the development of the self-flocculating phenotype in *Z. mobilis* , but the synthesis of

sufficient cellulose by the bacterial cells would be a prerequisite for them to self-flocculate, with significance in industrial applications.

Fig. 1

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 EAL activities, 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).

Table 1

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

Comparative genome analysis between ZM401 and ZM4 detected the SNP mutation in ZMO1055: thymine was 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 has been studied recently (Cao et al., 2022). However, little attention has been paid to the role of 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 (Fig. 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%, and the intracellular accumulation of c-di-GMP compromised less to 6.07 pg/mg protein, indicating that the SNP mutation substantially mitigated 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 (Fig. 2). Manipulation 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 a site-directed mutation of 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 out and the recombinant plasmid was transformed into ZM401, no significant change was observed in the intracellular accumulation of c-di-GMP, and the mutant ZM401ΔZMO1055^{-}/1055^{AAL} maintained the self-flocculating phenotype (Fig. 2). These experimental results indicate that the substitution of Ala526Val on ZMO1055^{+} in ZM401 compromised the protein's PDE activity for c-di-GMP degradation, as exhibited by the native gene bearing the AAL domain. This conclusion was validated by the reverse substitution of 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 (Fig. 2).

ZMO1055 also contains the GGDEF (GGDQF) domain, which catalyzes 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 (Fig. 2). These results indicate the DGC activity of ZMO1055 and 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.

Fig. 2

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 (0.76 pg/mg protein) was detected, indicating that ZMO0401 might function predominantly on c-di-GMP degradation (Fig. 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 to 28.2 from 9.8 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 production (Cao et al., 2022) (Fig. 3).

Fig. 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 and 10.04 pg/mg protein detected in the controls, and the self-flocculating phenotype of ZM401 was disrupted (Fig. 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 (Fig. 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 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 (Fig. 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 increasing to 46.1% and 30.1%, respectively, compared to only 5.5% observed in the control (Fig. 4B). These experimental results validated the catalytic function of the DGC domains in ZMO1365 and ZMO0919 in c-di-GMP biosynthesis.

Fig. 4

3.3 Engineering ZM4 with the self-flocculating phenotype

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

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

Cellulose fibrils have been validated as 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 to be 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. Therefore, we engineered ZM4 with the overexpression of ZMO1082-1083 and ZMO1082-1084 and the whole *bcs* operon ZMO1082-1085 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 efficiencies of 28.9%, 43.1% and 66.7%, respectively (Fig. 5A).

When both 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, a flocculating efficiency of 97.3% was observed for the bacterial cells, which was higher than that of 92.5% detected with ZM401 (Fig. 5A). Morphologies were further shown for ZM4 strains engineered with the deletion of ZMO1055, ZMO0401, and ZMO1487, overexpression of the *bcs* operon, and the combination of these two strategies (Fig. 5B).

Fig. 5

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 (Fig. S1), 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 costs 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* which have been intensively studied and delicately engineered as chassis cells (Mienda et al., 2021; Mitsui et al., 2021), much less is known about *Z. mobilis* being engineered as a microbial cell factory for robust production of bulk products, in particular through rational design.

As a signaling molecule, c-di-GMP regulates intracellular processes, including cellulose biosynthesis in bacteria (Ute et al., 2006; Jenal et al., 2012; Ross et al., 1987; Morgan et al., 2014). Therefore, understanding the biosynthesis and degradation of c-di-GMP in *Z. mobilis* is fundamental for its development 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 immobilization of bacterial cells within bioreactors. However, it will be beneficial to explore 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 specialized sensory and regulatory domains for responding to c-di-GMP, which accumulates intracellularly at different levels through a dynamic balance between its biosynthesis and degradation, and variants of enzymes with GGDEF and/or EAL domain(s) for DGC or/and PDE activities can fulfill such a task (Hengge et al., 2021; Petchiappan et al., 2020). ZMO1055, ZMO0401, ZMO1487, ZMO1365, and ZMO0919 are involved in c-di-GMP metabolism in ZM4 for the intracellular accumulation of c-di-GMP at different levels, but previous studies have confirmed that only ZMO0919 exhibits DGC activity (Jones-Burrage et al., 2015).

Our experimental results confirmed the function of ZMO0901, as reported previously (Jones-Burrage et al., 2015), and further validated that all four other genes are functional for c-di-GMP metabolism in ZM4. In addition to ZMO1055, which has been studied recently (Cao et al., 2022), the catalytic functions of ZMO0401, ZMO1487, and ZMO1365 on c-di-GMP metabolism in *Z. mobilis* were revealed for the first time. The reason for this discrepancy may be the different culture conditions. While rich medium was employed in our studies, minimal medium was used by Jones-Burrage et al. (Jones-Burrage et al., 2015), which could

affect the expression of genes and functions of encoded proteins. Therefore, these genes could be selected as targets for engineering to explore the role of c-di-GMP in metabolic regulation in *Z. mobilis* .

ZMO1365 and ZMO0919 enhanced c-di-GMP biosynthesis. Under the catalysis of DGC, c-di-GMP is synthesized from 2 mol of guanosine triphosphate (GTP) with 2 mol of diphosphate produced (Schirmer et al., 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 GTPase switch regulation (Wolff DW et al., 2022; Masuho et al., 2020; Cherfils et al., 2011), and also acts as a building block for synthesizing RNA during transcription (Attwater et al., 2018; Akoopie et al., 2020). Therefore, energy-intensive GTP production is finely regulated within cells to reduce the consumption of energy in the form of ATP. As a result, the overexpression of ZMO1365 and ZMO0919 in *Z. mobilis* to synthesize more c-di-GMP from GTP would not be an economic strategy for developing this species as a suitable chassis to be engineered as a microbial cell factory, since such a strategy could potentially affect intracellular processes involved with GTP.

Although ZMO1055 and ZMO0401 encode dual-functional proteins with both DGC and PDE domains to catalyze the biosynthesis and degradation of c-di-GMP, their PDE activities dominate the DGC activities for c-di-GMP degradation. Moreover, ZMO1487, with PDE activity, only catalyzes the degradation of c-di-GMP. Therefore, deactivating PDE activities by deleting ZMO1055, ZMO0401, and ZMO1487 would be preferred to compromise c-di-GMP degradation in *Z. mobilis* to enhance its intracellular accumulation, and consequently activate cellulose biosynthesis to flocculate bacterial cells (Xia et al., 2018; Morgan et al., 2014). These manipulations would exert less perturbation on other intracellular processes involved 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 a self-flocculating phenotype for applications from the viewpoint of bioprocess engineering. Thus, overexpression of the whole *bcs* operon composed of ZMO1082-1085 is needed for bacterial cells to synthesize sufficient amounts of cellulose under the regulation of c-di-GMP. Therefore, we proposed a strategy for engineering unicellular *Z. mobilis* strains with a self-flocculating phenotype through rational design (Fig. 6).

Fig. 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 biosynthesis and degradation catalyzed by enzymes with DGC and PDE activities activates the biosynthesis of cellulose fibrils for the bacterial cells to self-flocculate with significant advantages in industrial production. Compared to the 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 preferred for intracellular accumulation of the signal molecule, since this strategy would exert less perturbation on intracellular processes involved with or regulated by GTP. This study provides insights into the regulation of c-di-GMP on the morphological shift in *Z. mobilis* from unicellular cells to multicellular flocs, as well as guidelines for engineering unicellular strains from this species and other bacteria with the self-flocculating phenotype through rational design. Moreover, this study provides a basis for exploring the mechanism underlying c-di-GMP metabolism in *Z. mobilis* and its regulation of other intracellular processes.

Competing interests

The authors declare no competing interests.

Author contributions

K.L. drafted the manuscript. J. X. performed all the experiments and performed a preliminary analysis of the experimental results. C.G.L. and F.W.B. supervised this study.

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Data availability

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

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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	Cyclase/phosphodiesterase for c-di-GMP synthesis/degradation		579
ZMO0401	Cyclase/phosphodiesterase for c-di-GMP synthesis/degradation		683
ZMO1487	Phosphodiesterase for c-di-GMP degradation		258
ZMO0919	Cyclase for c-di-GMP synthesis		1008
ZMO1365	Cyclase for c-di-GMP synthesis		1045

Beta-propeller signal transduction domain; Periplasmic ligand-binding sensor domain;

GGDEF domain for cyclase activity; EAL domain for phosphodiesterase activity;

Domain for transmembrane helices; ORF: open reading frame; aa: amino acids.

Figure Legends

Fig. 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.

Fig. 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 doman 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 delection 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 delection 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.

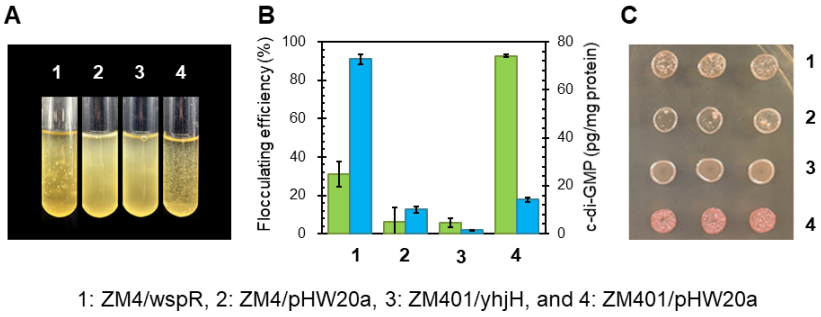
Fig. 3 Contribution of ZMO0401 to self-flocculation of the bacterial cells of *Z. mobilis* () and intracellular accumulation of c-di-GMP () (A), and morphologies observed in flask cultures when shaking was stopped for 3-5 seconds (B). / and Δ: gene overexpression and deletion. ZM4/pHW20 and ZM401/pHW20: ZM4 and ZM401 engineered with the empty vector pHW20a as the controls.

Fig. 4 Impact of the deletion (Δ) and overexpression (/) of ZMO1487 (a), 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.

Fig. 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 ().

Fig. 6 Strategies for engineering unicellular *Z. mobilis* strains with the self-flocculating phenotype through enhancing intracellular accumulation of c-di-GMP to activate cellulose biosynthesis (genes in bold and plain fonts are for deletion and overexpression, respectively).

Figure 1



1: ZM4/wspR, 2: ZM4/pHW20a, 3: ZM401/yhjH, and 4: ZM401/pHW20a

Figure 2

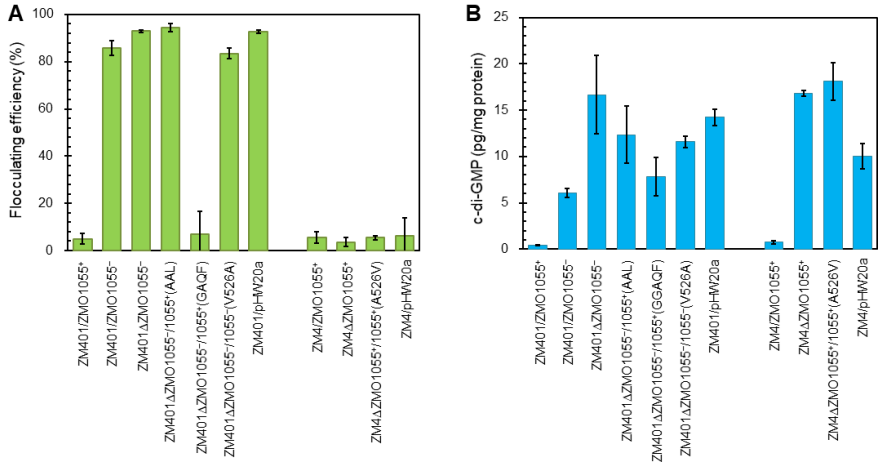


Figure 3

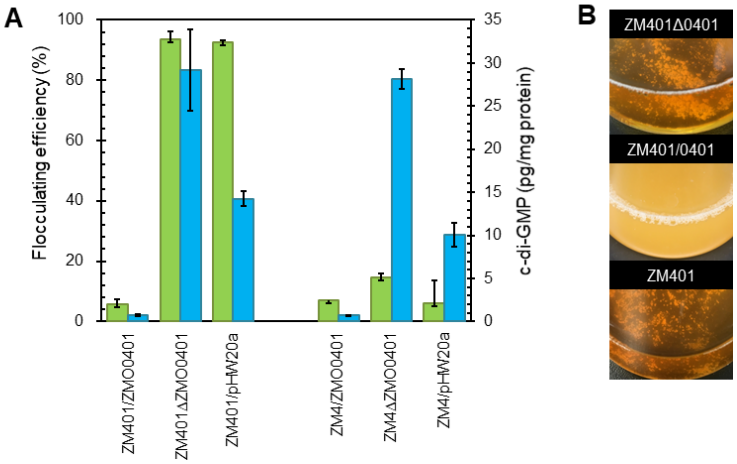


Figure 4

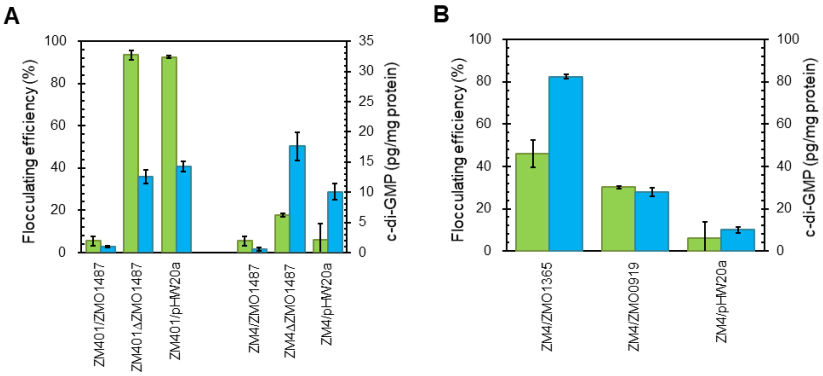


Figure 5

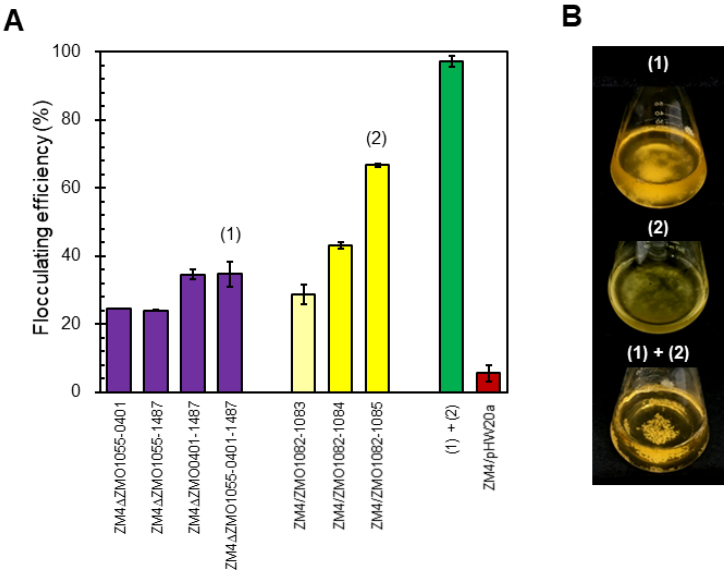


Figure 6