Trends in Biotechnology

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Review

Engineering *Zymomonas mobilis* for Robust Cellulosic Ethanol Production

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Great effort has been devoted to engineering *Saccharomyces cerevisiae* with pentose metabolism through the oxido-reductase pathway for cellulosic ethanol production, but intrinsic cofactor imbalance is observed, which substantially compromises ethanol yield. *Zymomonas mobilis* not only can be engineered for pentose metabolism through the isomerase pathway without cofactor imbalance but also metabolizes sugar through the Entner–Doudoroff pathway with less ATP and biomass produced for more sugar to be used for ethanol production. Moreover, the availabilities of genome sequence information for multiple *Z. mobilis* strains and advanced genetics tools have laid a solid foundation for engineering this species, and the self-flocculation of the bacterial cells also presents significant advantages for bioprocess engineering. Here, we highlight some of recent advances in these aspects.

Highlights

Zymomonas mobilis metabolizes glucose through the Entner–Doudoroff (ED) pathway, with less ATP generated and biomass accumulated for more ethanol production.

The large specific cell surface of *Z. mobilis* together with the ED pathway facilitates glucose uptake and ethanol fermentation.

Its metabolic characteristics and narrow substrate spectrum make *Z. mobilis* unsuitable for fuel ethanol production from sugar- and starch-based feedstocks, but it would be a good host to be engineered for cellulosic ethanol production.

When self-flocculated, *Z. mobilis* can be immobilized within fermenters for high cell density to improve ethanol productivity. Meanwhile, its tolerance to environmental stresses may be enhanced by this morphological change.

Both *Z. mobilis* ZM4 and its self-flocculating mutant ZM401 can tolerate more than 100 g/L ethanol, which is sufficient for cellulosic ethanol production.

Workhorses for Ethanol Production

Fuel ethanol is currently produced from sugar- and starch-based feedstocks and is called first-generation (1G) fuel ethanol, but considering the increasing global population and demand for food supply, it is not practical to produce enough 1G fuel ethanol to fulfill the goal of sustainable transportation fuels by alleviating dependence on crude oil. Furthermore, debates about the impacts of 1G fuel ethanol production on food security have persisted throughout the past decade [1]. Lignocellulosic biomass, particularly agricultural residues, is non-food related and abundantly available, and it represents a sustainable feedstock for producing second-generation (2G) fuel ethanol [2]. Motivated by the 1970s energy crisis, great effort has been devoted to cellulosic ethanol since then, and pilot and demonstration plants have been established to test its techno-economic viability, but unfortunately 2G fuel ethanol is still not economically competitive for large-scale commercial production [3,4].

Microbial strains are workhorses for ethanol production. Strains of *Saccharomyces cerevisiae* dominate ethanol fermentation from sugar- and starch-based feedstocks, but they are not suitable for cellulosic ethanol production because they cannot ferment pentose sugars released during the hydrolysis of hemicelluloses in lignocellulosic biomass [5,6]. This drawback not only compromises ethanol yield but also increases workload for stillage treatment. However, *S. cerevisiae* can convert xylulose to xylulose 5-phosphate, an intermediate of the **pentose phosphate pathway** (see Glossary) to be further metabolized to glyceraldehyde 3-phosphate for ethanol production through the glycolysis pathway. Therefore, *S. cerevisiae* can be engineered with xylose metabolism through the heterologous expression of genes encoding key enzymes in pentose-utilizing microorganisms such as *Scheffersomyces stipitis* [7], together with the overexpression of xylulokinase, for the cofermentation of pentose and hexose sugars to produce ethanol.

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S. stipitis converts xylose through its oxido-reductase pathway [8], through which xylose reductase (XR) reduces xylose to xylitol that is then oxidized to xylulose by xylitol dehydrogenase (XDH). Therefore, engineering *S. cerevisiae* with the oxido-reductase pathway is preferred. However, cofactors for XR and XDH cannot be regenerated for balance in engineered *S. cerevisiae* because **NADPH/NADP$^+$** is required by XR and **NAD$^+$/NADH** is needed for XDH (Figure 1). The intrinsic imbalance of cofactors results in xylitol accumulation [9]. Engineering cofactor preference for the enzymes has thus been performed, but the progress is slow. Recently, an alternative strategy has garnered more attention, in which expression of a heterologous xylose isomerase (XI) is explored, but the XI pathway is present mainly in bacteria and seems less efficient in *S. cerevisiae* [10].

The rod-shaped bacterium *Zymomonas mobilis* is a Gram-negative, facultatively anaerobic and nonsporulating ethanologen that metabolizes sugar through the **Entner–Doudoroff (ED) pathway** in conjunction with the key enzymes pyruvate decarboxylase and alcohol dehydrogenase for ethanol production [11–13]. As shown in Figure 2, the ED pathway is different from the **Embden–Meyerhof–Parnas (EMP) pathway** in *S. cerevisiae*. For the EMP pathway, energy generated in the form of **ATP** needs to be dissipated through yeast growth, and ethanol

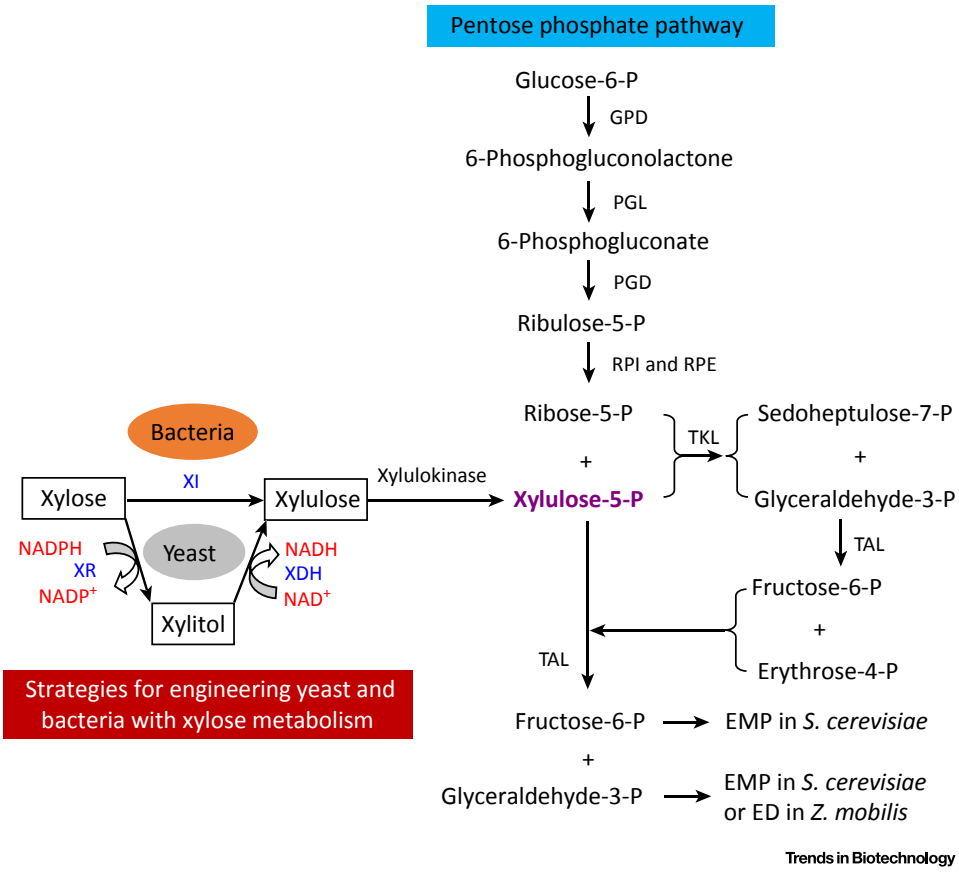

Figure 1. Strategies for Engineering *Saccharomyces cerevisia* and *Zymomonas mobilis* with Xylose Metabolism for Cellulosic Ethanol Production. Abbreviations: ED, Entner–Doudoroff pathway; EMP, Embden–Meyerhof–Parnas pathway; GPD, glucose-6-phosphate dehydrogenase; PGD, 6-phosphogluconate dehydrogenase; PGL, 6-phosphogluconolactonase; RPE, ribulose-5-phosphate epimerase; RPI, ribulose-5-phosphate isomerase; TAL, transaldolase; TKL, transketolase; XDH, xylitol dehydrogenase; XI, xylose isomerase; XR, xylose reductase.

Glossary

ATP: a high-energy compound for metabolism. When consumed, it converts to ADP or further to AMP.
Bacterial cellulose synthase: a membrane-integrated complex composed mainly of the inner membrane components BcsA and BcsB and the outer membrane protein BcsC that function together for cellulose synthesis and translocation.
Bioreactor hydrodynamics: flow, shearing, and mixing within bioreactors, a bioprocess engineering strategy for controlling the size of microbial flocs.
Chemostat: the unique property for bioreactors operated under continuous conditions in which cell growth is automatically balanced by washing with the effluent, and cell growth can be controlled by the flow rate of medium (dilution rate).
Entner–Doudoroff (ED) pathway: an alternative glycolysis for converting glucose to pyruvate with distinct features for the formation of 2-keto-3-deoxy-6-phosphogluconate as the key intermediate and the bifurcated routes for its metabolism with much less ATP produced.
Embden–Meyerhof–Parnas (EMP) pathway: the common strategy for glycolysis that converts glucose to pyruvate through energy consumption and production processes, with a net energy efficiency of two ATP released per glucose consumed.
Fermentation kinetics: equations describing substrate consumption, cell growth, and product formation, providing a strategy for controlling the self-flocculation of *Z. mobilis* within bioreactors through controlling CO_2 production during ethanol fermentation.
Immobilized/self-immobilized cells: when cells are constrained by supporting materials or membranes, they cannot be washed away with the effluent for high density within bioreactors (immobilized cells). The same purpose can be achieved when cells are self-flocculating (self-immobilized cells).
NAD$^+$/NADH: a cofactor for intracellular redox reactions that presents in the oxidized and reduced forms. While NAD$^+$ accepts electrons from electron donators, NADH

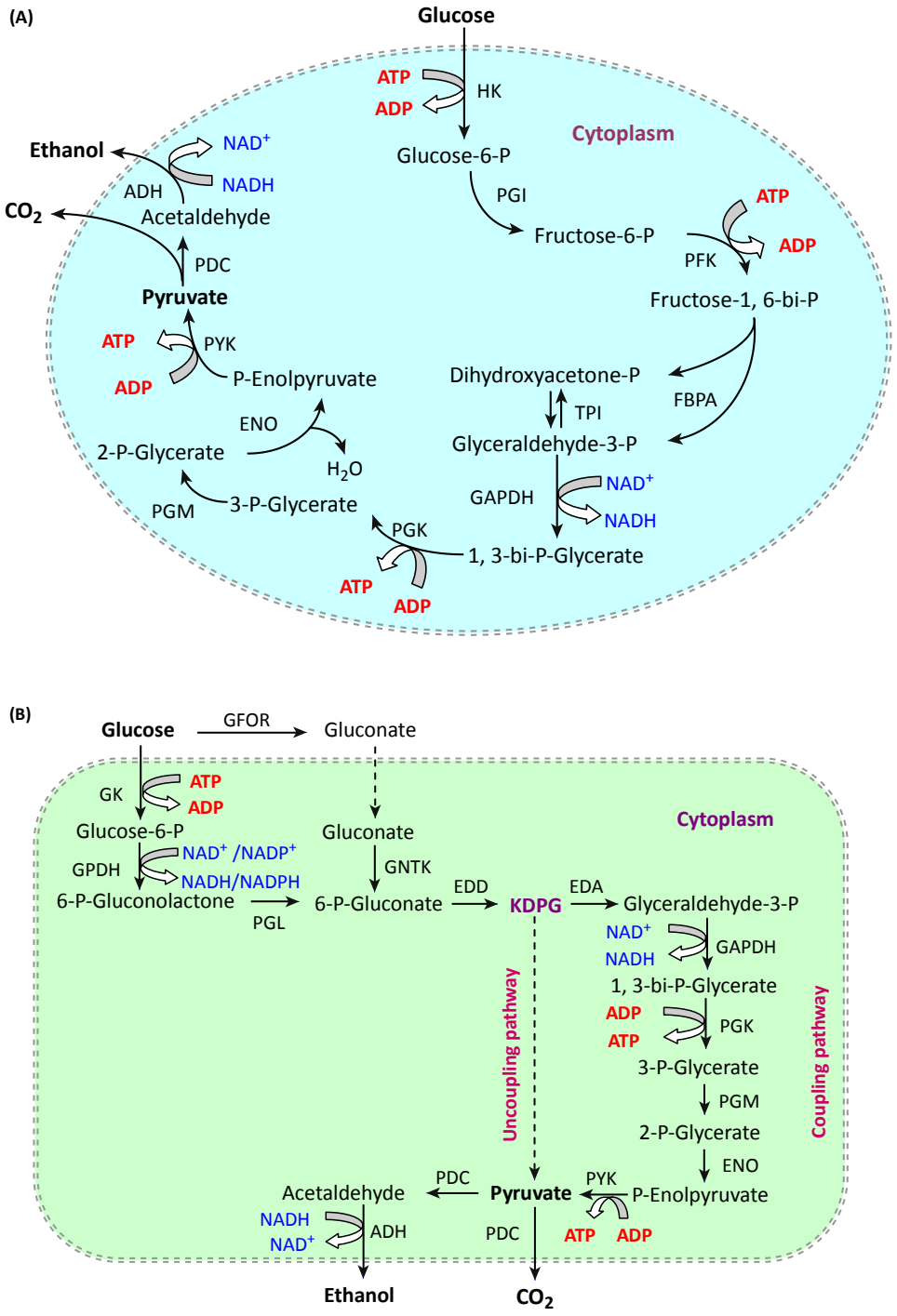

Figure 2. Glycolysis Pathways for *Saccharomyces cerevisiae* and *Zymomonas mobilis*. Glycolysis for *S. cerevisiae* through the Embden–Meyerhof–Parnas pathway (A) and for *Z. mobilis* through the Entner–Doudoroff pathway (B). Abbreviations: ADH, alcohol dehydrogenase; EDA, 2-keto-3-deoxy-gluconate aldolase; EDD, 6-phosphogluconate

(Figure legend continued on the bottom of the next page.)

donates electrons to electron acceptors.

NADP$^+$/NADPH: a cofactor mainly for anabolic metabolism. Similar to NAD$^+$/NADH, it also presents in the oxidized and reduced forms (NADP$^+$/NADPH).

Observed yield: a yield that is calculated based on substrate feeding into bioreactors, without deduction of unconverted substrate. For ethanol fermentation, observed yield is calculated based on total sugars in the feedstock.

Pentose phosphate pathway: an alternative pathway to glycolysis, particularly for anabolic metabolism, to provide phosphorylated ribose as the backbone for the biosynthesis of genetic materials such as DNA and RNA. When intermediates such as glyceraldehyde 3-phosphate are over-accumulated, they can be directed to glycolysis for catabolic metabolism.

Quasi-steady state: an operational mode for a bioreactor operated under continuous conditions, at which parameters including concentrations of substrate, biomass, and product are relatively stable.

Quorum sensing (QS): process for cell-to-cell communication among bacteria for a collective response to environmental stresses, which occurs under high cell density culture conditions for autoinducers or signal molecules secreted by individual cells to approach thresholds to trigger this phenomenon.

Box 1. How Can *Z. mobilis* Produce More Ethanol with High Productivity?

Although less biomass is accumulated through the ED pathway in *Z. mobilis*, improved ethanol yield and productivity might not be achieved if biomass density is too low during ethanol fermentation, since prolonged fermentation time is needed. For example, experimental results indicated that under batch fermentation conditions, even though much less biomass accumulation of 3.3 g/L was achieved with *Z. mobilis* ZM4, compared with that of 7.1 g/L achieved with *S. cerevisiae* Angel Super ADY, no significant improvement in ethanol yield was observed when medium containing ~200 g/L glucose was used, and ethanol fermentation was completed at 50 h [17]. However, when the fed-batch strategy was adopted to mitigate glucose inhibition in *Z. mobilis* ZM4 and facilitate the fermentation process, the fermentation time was reduced to 30 h with 98.6 g/L ethanol produced and 5.5 g/L biomass accumulated from 204.0 g/L glucose consumed, and an increase of 2.1% in ethanol yield was observed compared with that achieved by *S. cerevisiae* Angel Super ADY and *Z. mobilis* ZM4 as well under batch fermentation conditions [17]. The reason for low ethanol productivity under low biomass density conditions is apparent, but why ethanol yield was increased when the fermentation time was reduced and more biomass was accumulated needs to be explored for *Z. mobilis* from the viewpoint of scientific fundamentals.

production is coupled with biomass accumulation. While in the ED pathway, there are bifurcated routes for metabolizing the key intermediate 2-keto-3-deoxy-6-phosphogluconate directly to pyruvate without ATP production or indirectly to pyruvate with ATP generated, making ethanol production partly decoupled from cell growth. Compared with the EMP pathway, 50% less ATP is produced through the ED pathway. As a result, less biomass is accumulated during ethanol fermentation by *Z. mobilis* [14]; consequently, more sugar can be fueled for ethanol production to increase its **observed yield** (Box 1). Moreover, the bacterial cells are $1-2\times2-6$ μm in size, much smaller than those of *S. cerevisiae* ($2-10\times4-20$ μm), so a large surface area is available for glucose uptake [11]. The large surface area and the ED pathway give the bacterium the nickname 'catabolic highway' [15,16]. One representative study compared ethanol fermentation of *Z. mobilis* ZM4 and *S. cerevisiae* by using medium composed of ~200 g/L glucose in which 3.30 g/L biomass was accumulated with ZM4, less than 50% of that accumulated by the yeast, but the two strains completed ethanol fermentation at the same time of 50 h, and the specific ethanol production rate was doubled with the bacterium [17].

However, *Z. mobilis* metabolizes only glucose, fructose, and sucrose, and the ethanol yield from sucrose is substantially compromised due to the formation of levan [15], making it not suitable for ethanol production from sugarcane juice or molasses. Meanwhile, grains with starch as the major carbohydrate are used in industry, and starch needs to be hydrolyzed into sugars. Although glucose is the major sugar, there are other sugars such as maltose and maltotriose in the hydrolysate that are fermentable to *S. cerevisiae*, but not to *Z. mobilis*. Therefore, *Z. mobilis* cannot be used for ethanol production from grains. Cellulosic ethanol production has created an opportunity for exploring the advantages of *Z. mobilis*. Lignocellulosic biomass is composed mainly of cellulose, hemicelluloses and lignin, and glucose is the only sugar released from cellulose hydrolysis. As a result, the issue of narrow substrate spectrum with *Z. mobilis* for ethanol production from sugar and grains is no longer a problem for cellulosic ethanol production.

Morphologies of *Z. mobilis* and Their Impacts on Ethanol Production

In addition to unicellular cells, self-flocculation has been observed for *Z. mobilis* ZM401, a mutant of *Z. mobilis* ZM4, through which the bacterial cells aggregate to form flocs (Figure 3).

dehydratase; ENO, enolase; FBPA, fructose-1,6-bisphosphate aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFOR, glucose–fructose oxidoreductase; GK, glucokinase; GNTK, gluconate kinase; GPDH, glucose-6-phosphate dehydrogenase; HK, hexokinase; KDPG, 2-keto-3-deoxy-6-phosphogluconate; PDC, pyruvate decarboxylase; PFK, phosphofructokinase; PGI, phosphoglucoisomerase; PGK, phosphoglycerate kinase; PGL, phosphogluconolactonase; PGM, phosphoglyceromutase; PYK, pyruvate kinase; TPI, triose phosphate isomerase.

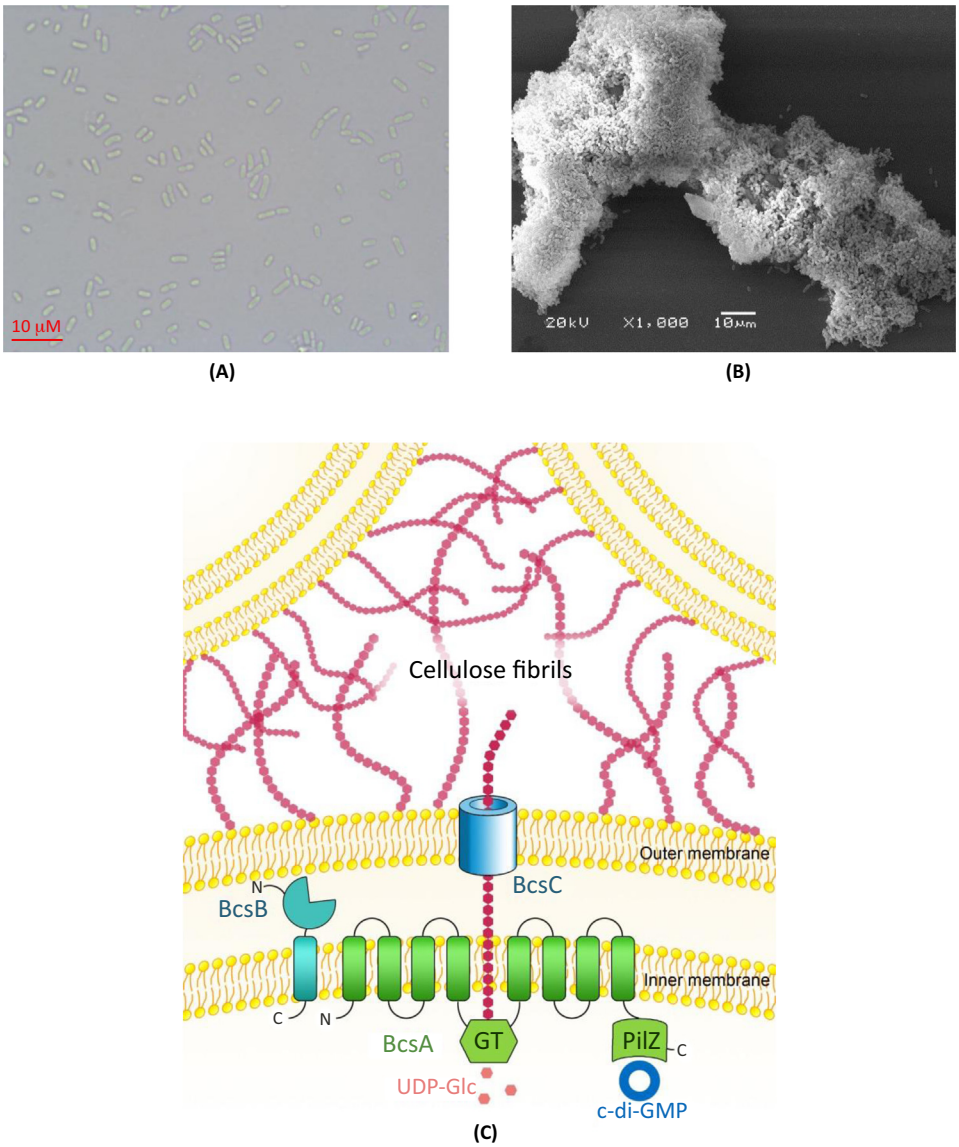

Figure 3. Morphologies of *Zymomonas mobilis*. The unicellular cells of *Z. mobilis* ZM4 (A), the self-flocculating morphology of *Z. mobilis* ZM401 (B), and the synthesis of cellulose fibrils for the self-flocculation of the bacterial cells (C). Adapted with permission from [17,23]. Abbreviations: BcsA-C, subunits A-C of bacterial cellulose synthase; C, C terminal; c-di-GMP, cyclic diguanylate; GT, glucosyltransferase; N, N terminal; PilZ, c-di-GMP binding domain; UDP-Glc, uracil-diphosphate glucose.

Chemostat for Continuous Ethanol Production

Compared with batch process, continuous fermentation is preferred for fuel ethanol production at large scales for high productivity. When unicellular cells are used, their growth within fermenters is automatically balanced by their leaving with the effluent under **chemostat** conditions, and the dilution rate of substrate controls their specific growth rate, which is limited ultimately by the maximal specific growth rate (Figure 4A). Therefore, high biomass density

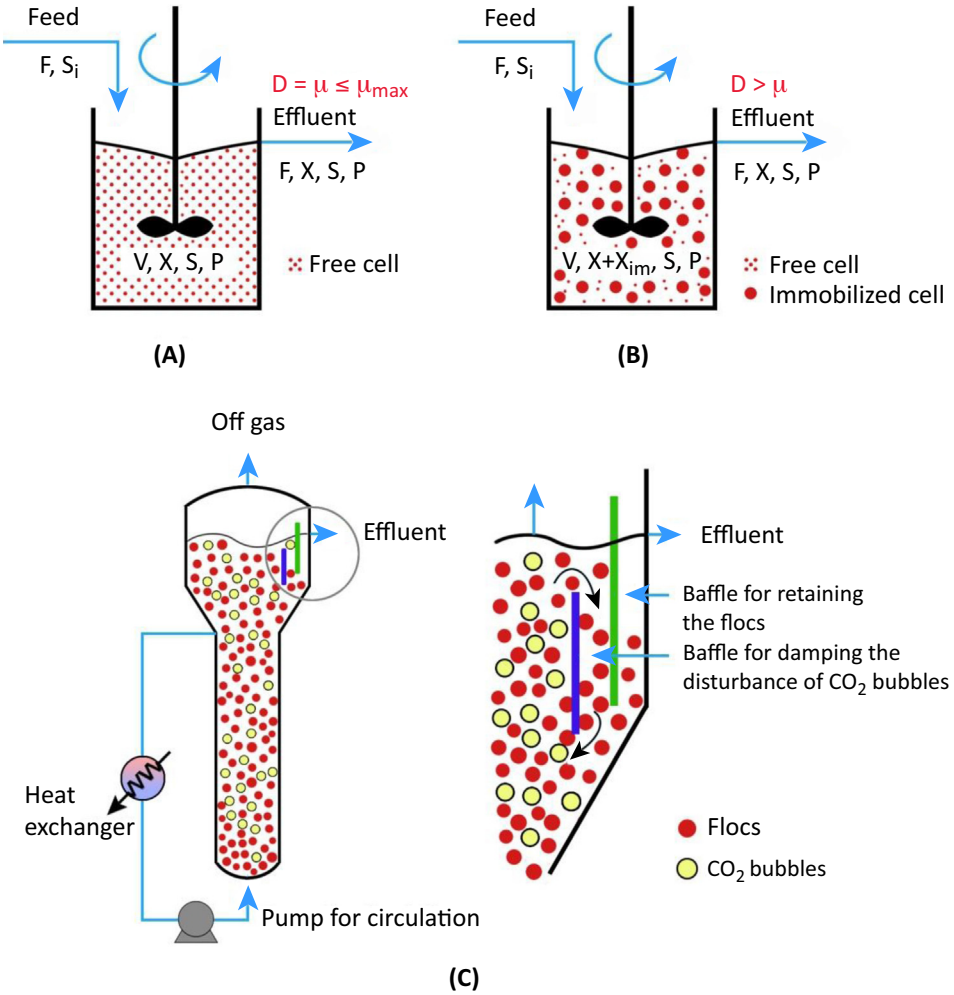

Figure 4. Chemostat and Quasi-Steady State for Continuous Ethanol Production. (A) Continuous culture and fermentation with unicellular cells at chemostat conditions, at which the specific cell growth rate μ is automatically controlled by the dilution rate D = F/V, where F and V are the flow rate of medium and working volume of the bioreactor, respectively, but D is ultimately limited by the maximal specific growth rate $μ_{max}$. (B) Immobilized cells using supporting materials or membranes through which the correlation between μ and D developed under the chemostat conditions is decoupled. S_i and S are the concentrations of limiting substrate in the medium and the effluent, respectively; X and X_{im} are the concentrations of biomass freely suspended and immobilized within bioreactors, respectively; and P is the product concentration in the effluent. (C) Self-immobilized cells through their self-flocculation without consumption of supporting materials or using membranes. Modified with permission from [19].

cannot be accumulated within fermenters. The solution for this issue is using **immobilized cells**. Although cells can be immobilized using supporting materials or through membrane retention (Figure 4B), they are not scientifically solid and economically competitive. On the one hand, ethanol is a primary metabolite, and its production is coupled with the growth of *S. cerevisiae* through the EMP pathway or partly coupled with the growth *of Z. mobilis* through the ED pathway. When cells are immobilized by supporting materials, their growth is constrained by physical limitation, making them not productive for ethanol production. On the other hand, fuel ethanol is a bulk commodity with low market prices, and extra costs with the consumption of

supporting materials and the preparation of immobilized cells at large scales are unacceptable to the industry.

Immobilization of *Z. mobilis* Cells through Self-Flocculation

When *Z. mobilis* is self-flocculating, the bacterial cells can be immobilized within fermenters and are termed **self-immobilized cells**. This advantage of *Z. mobilis* was explored for ethanol production from glucose in the 1980s, and tank fermenters with external settlers were developed through which high cell densities were accumulated under continuous ethanol fermentation conditions to improve ethanol productivity [18]. Moreover, column fermenters with expanded upper sections and internal separation configurations were designed for continuous ethanol fermentation by using self-immobilized yeast cells [19], which would be more suitable for continuous ethanol fermentation by the bacterial flocs at large scale than tank fermenters (Figure 4C). It is worth noting that when microbial cells are immobilized within fermenters through their self-flocculation, regardless of what kind of microorganisms, *Z. mobilis* or *S. cerevisiae*, chemostat observed with unicellular cells cannot be established, because microbial flocs cannot be washed away freely with the effluent to balance their growth as that is observed with unicellular cells, and biomass is accumulated within the fermenters. However, this issue can be addressed by purging biomass periodically to control its density within the fermenters at designated levels for ethanol fermentation to be performed with industrial standards, an operational mode called **quasi-steady state**.

Other Advantages of *Z. mobilis* Self-Flocculation

Another advantage with the self-flocculation of *Z. mobilis* is the potential for enhanced tolerance to stresses, and fundamentals underlying this phenomenon might be **quorum sensing** (QS), which has been observed in other Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* [20]. Compared with the unicellular morphology of *Z. mobilis*, the bacterial flocs characterized by cell-to-cell contact are the upper limit for localized high cell density that is required for triggering QS. This physiological trait is extremely important for bioethanol production, since toxic byproducts such as acetic acid, furans, and phenolic compounds are released during the pretreatment of lignocellulosic biomass. Although various detoxification technologies have been developed, none of them are economically feasible [21]. When ethanol fermentation was performed with *Z. mobilis* ZM4 and its self-flocculating mutant *Z. mobilis* ZM401 by using medium supplemented with furfural, hydroxymethyl furfural (HMF), acetic acid, and vanillin, respectively, improved tolerance to acetic acid and vanillin was observed for the mutant and consequently more ethanol was produced [17].

Moreover, when ethanol fermentation is complete, the self-flocculation of *Z. mobilis* facilitates biomass recovery through cost-effective gravity sedimentation instead of centrifugation with high capital investment in centrifuges and intensive energy consumption for their operation or membrane separation with a challenge of membrane fouling. In case of small-scale ethanol production, particularly for cellulosic ethanol production that might be limited by feedstock logistics, and a batch process is preferred, the repeated batch fermentation can be performed with the self-flocculating *Z. mobilis* through purging supernatant, but with the bacterial flocs retained as inoculum to start the next operation quickly by replenishing the fermenter with fresh substrate.

Challenges in Bioprocess Engineering for Substrate Preparation

When *Z. mobilis* is self-flocculating, it presents special requirements for bioprocess engineering, and clear substrate without solid residues should be supplied so that the fermenters can be operated at quasi-steady state. Otherwise, if substrate with solid residues is fed, the solid residues accumulate quickly within the fermenters to disrupt the quasi-steady state developed

Box 2. What Process Is Better for Cellulosic Ethanol Production?

Two different processes, separate hydrolysis and cofermentation (SHCF) and simultaneous saccharification and cofermentation (SSCF), have been developed for cellulosic ethanol production from separate hydrolysis and fermentation and simultaneous saccharification and fermentation (SSF) previously developed for ethanol production from grains [62]. For SHCF, hydrolysis of the cellulose and hemicelluloses is performed completely before ethanol fermentation, and the enzymatic reactions are operated at optimal conditions, particularly at ~50 °C, a temperature that is optimal to cellulases and hemicellulases in case they are also supplemented [63]. Another advantage with SHCF is that lignin can be removed by filtration from the hydrolysate, and sugar concentration can be increased for high-gravity fermentation to increase ethanol titer, saving energy consumption for ethanol recovery by distillation, and also reducing stillage discharge. Moreover, ethanol fermentation can be performed with less energy consumption for stirring and mixing without obstruction from lignin [64]. The disadvantage with SHCF is that a small amount of sugar is lost with the lignin cake. As for SSCF, the hydrolysis/saccharification of the cellulose and hemicelluloses and ethanol fermentation are carried out under the same conditions, particularly at the low temperature of 30–37 °C for ethanol fermentation by either *S. cerevisiae* or *Z. mobilis*, much lower than that for cellulose hydrolysis by cellulases [62]. Although the word 'simultaneous' has been used to describe SSCF for a long time, the two processes are by no means simultaneous, since sugars released from the hydrolysis are consumed sequentially for ethanol production. The biggest advantage for SSCF is its low risk for contamination, because almost no sugars are accumulated, but high ethanol titer cannot be achieved due to the presence of lignin. Unlike ethanol production from grains in which SSF presents more advantages for commercial production, no agreement on which process, SHCF or SSCF, is more suitable for cellulosic ethanol production has been achieved. However, it is clear that only SHCF is suitable for cellulosic ethanol production by *Z. mobilis* with the self-flocculating phenotype.

for the bacterial flocs. For lignocellulosic biomass, clear substrate is available through process arrangements for the enzymatic hydrolysis of the cellulose component and ethanol fermentation of the hydrolysate (Box 2).

Self-Flocculation Control for *Z. mobilis*

When the bacterial flocs are used for cellulosic ethanol production, their sizes should be controlled properly. The larger the flocs are, the better the performance of their gravity sedimentation will be for separation from the effluent; however, mass transfer limitations may occur for nutritional components to be transported to the inner of the bacterial flocs. The self-flocculation of *Z. mobilis* can be controlled properly through genetic manipulations of the strains and bioprocess engineering for production. Strain manipulations depend on elucidating the molecular mechanism underlying the self-flocculation of microbial cells, such as what has been done for the self-flocculating *S. cerevisiae* [19]. For example, the self-flocculation of yeast cells can be controlled at the molecular level by editing the number of intragenic repeats in the gene *FLO1* to control the biosynthesis of sugar residues because glycoproteins are chemicals for their self-flocculation, and sugar residues are ultimately responsible for the phenotype [22]. However, this fundamental work just started for *Z. mobilis* with the identification of cellulose fibrils as the basis for its self-flocculation [23]. Once the molecular mechanism underlying the biosynthesis of cellulose fibrils is further deciphered, the self-flocculation of *Z. mobilis* can be engineered at molecular levels.

Bioprocess engineering can control the self-flocculation of microbial cells directly through **bioreactor hydrodynamics** and **fermentation kinetics**, since the self-flocculation of microbial cells is coordinated through weak forces that can be counteracted easily by the shearing force created through mixing and flow within bioreactors as well as by the swelling force caused from CO_2 accumulated within the microbial flocs during ethanol fermentation with either *S. cerevisiae* or *Z. mobilis*, which are also highlighted in the control of the self-flocculation of *S. cerevisiae* [19].

Engineering *Z. mobilis* for Cellulosic Ethanol Production

*Z. mobilis*cannot utilize pentose sugars, which must be engineered with pentose metabolism for cellulosic ethanol production. However, the strategy for engineering the bacterium with

pentose metabolism is the heterologous expression of isomerase to convert pentose such as xylose directly to xylulose that can be further metabolized by its pentose phosphate pathway for ethanol production (Figure 1). In addition, a better understanding of its physiology such as nutritional requirements and stress responses is needed for industrial applications.

Cofermentation of Pentose and Hexose Sugars

The first *Z. mobilis* strain for xylose utilization was developed at the National Renewable Energy Laboratory (NREL) through the heterologous expression of genes encoding xylose isomerase (*xylA*), xylulokinase (*xylB*), transaldolase (*tal*), and transketolase (*tktA*) [24]. Soon after, a similar strategy was applied to engineer *Z. mobilis* with arabinose metabolism [25]. The challenge in engineering *S. cerevisiae* with pentose metabolism has created opportunities for exploring the merits of *Z. mobilis*. Although rational designs for engineering *Z. mobilis* are still premature, semirational strategies have been developed. For example, expression of a xylose-specific transporter XlyE from *E. coli* in *Z. mobilis* facilitated xylose transport [26], and by using arabinose as the sole carbon source, a mutant to metabolize arabinose in the hydrolysate of corn stover pretreated by diluted acid more efficiently was selected [27]. Other cases are the screening of mutants through the laboratory-directed evolution of *Z. mobilis* with increased xylose concentrations and in the presence of 2-deoxyglucose for more efficient fermentation of xylose in the rich medium and hydrolysate of pretreated corn stover, respectively [28,29], and high-throughput sequencing can then be applied to the mutants to pinpoint genetic mutations responsible for the phenotypes of the adapted strains for engineering strains through rational designs [30].

Unless pretreated with ammonia, the hydrolysate of lignocellulosic biomass lacks nutritional components, particularly a nitrogen source. Genomic analysis of *Z. mobilis* provides insight on genes encoding nitrogenases for N_2 fixation to provide a nitrogen source [31], as supported by experimental work [32], but it is not useful for cellulosic ethanol production under anaerobic conditions, since no nitrogen gas is available without aeration. By contrast, diammonium phosphate, which provides a source of both nitrogen and phosphorus, can be supplemented. If ammonia is used to neutralize the pretreated biomass, or ammonia fiber explosion/expansion pretreatment is applied for lignocellulosic biomass [33,34], the remaining ammonia can be used as the nitrogen source. Nevertheless, nutritional optimization is needed for cellulosic ethanol production by *Z. mobilis*.

Development of Stress-Tolerant Strains

The genome of *Z. mobilis* is relatively small [35], which may make it less effective in stress response. However, *Z. mobilis* can ferment media containing glucose at ~200 g/L with more than 90 g/L ethanol produced [17], which is sufficient for cellulosic ethanol production, since hydrolysate with total sugars more than 200 g/L cannot yet be prepared economically from lignocellulosic biomass. Studies on ethanol tolerance are important for *Z. mobilis*, but priority should be devoted to improving its tolerance to inhibitors released during the pretreatment of lignocellulosic biomass.

Acetic acid is a predominant byproduct and inhibition occurs from its undissociated form at pH < 4.5 for ethanol fermentation, which can diffuse across plasma membranes by facilitated diffusion [21]. Under nearly pH-neutral cytosolic environments, acetic acid dissociates, resulting in acidification of the cytoplasm and disruption of intracellular metabolism [36]. The inhibitory effect of acetic acid was analyzed using *Z. mobilis* 8b previously engineered with xylose metabolism [24], and expression profiles of genes related to biosynthesis, glycolysis, energy metabolism, and response to acetic acid were characterized [37], which would provide insight

on directions of further research. Moreover, adaption under acetic acid stress is still an effective strategy for selecting tolerant mutants for studies to identify target genes and pathways for rational designs, as was highlighted by the adaption of *Z. mobilis* 8b on diluted acid-pretreated corn stover hydrolysate [38].

Furfural and HMF are derived from the degradation of pentose and hexose, and phenolic compounds including vanillin, coniferyl aldehyde, and 4-hydroxybenzoic acid are products of lignin breakup; these compounds are also inhibitors presented in the hydrolysate of lignocellulosic biomass [21]. Studies on *S. cerevisiae* have indicated that mechanisms underlying the inhibition of these compounds are mainly from their hydrophobicity for selective attack on cell membranes to disrupt their integrity that is required for physiological functions; damage of the synthesis of macromolecules including DNA, RNA, and proteins for growth; and inhibition of key enzymes that are essential to central metabolism such as alcohol dehydrogenases for ethanol production [39–41]. Although studies on the impact of inhibitors on *Z. mobilis* are lagging behind those on *S. cerevisiae*, similar mechanisms may exist for the bacterium to respond to the inhibition of those inhibitors.

Z. mobilis can metabolize furans through reducing them to less toxic alcohols when their concentrations are below thresholds for complete inhibition, thereby providing a strategy for engineering the bacterium with an overexpression of native dehydrogenase, together with cofactor regeneration, to enhance its tolerance to furans [42]. In addition, oxidation of furfural and HMF through heterologous overexpression of peroxidases such as manganese peroxidase (MnP) may be a strategy for *Z. mobilis* to detoxify furans without a necessity for cofactor regeneration [43]. Interestingly, overexpression of MnP, one of the major lignolytic enzymes in fungi and bacteria for lignin degradation [44], also benefits the detoxification of phenolic compounds, but the radical-mediated reaction may generate oxidative stress on *Z. mobilis*. Moreover, random mutagenesis strategies such as radiation, chemical treatment, adaptive evolution, and genome shuffling as well as their combination have been applied to *Z. mobilis* to generate mutants with enhanced tolerance to furans for exploring underlying mechanisms for rational design [45,46].

Phenolic compounds such as vanillin, coniferyl aldehyde, *p*-coumaric acid, and ferulic acid as well as nonphenolic aromatic compounds including benzoic acid and benzyl alcohol are also present in the hydrolysate of lignocellulosic biomass. They are derived from the degradation of lignin and other reactions occurring among chemicals released during pretreatment, but their concentrations depend on the characteristics of lignocellulosic biomass and pretreatment methods, and are relatively low due to the recalcitrance of lignin to degradation [21]. Moreover, when pretreatment technologies are developed, lignin should not be degraded completely into inhibitory byproducts but maintained intact or fragmented properly for valorization to credit cellulosic ethanol production [47,48]. Although their impact would be less severe than acetic acid, furfural, and HMF, phenolic compounds are still toxic, as was shown during ethanol fermentation by *S. cerevisiae* [49]. However, when their concentrations are lower than thresholds for inhibition, coniferyl aldehyde, *p*-coumaric acid, and ferulic acid can be detoxified by *S. cerevisiae* through metabolism [50,51], and similar phenomena may also occur in *Z. mobilis*. For example, one study highlighted ethanol fermentation of the hydrolysate of corncob residue left after xylitol production, and the mechanism underlying the detoxification was explored through a transcriptomic study [52,53], indicating that *Z. mobilis* might be more tolerant to phenolic compounds than *S. cerevisiae*. However, more solid experimental data and theoretical analysis are needed.

Genome-Guided Systems Approaches for Strain Development

Strain development with *Z. mobilis* has been focused mainly on engineering it with pentose metabolism and tolerance to inhibitors for cellulosic ethanol production under stressful conditions through bottom-up strategies. As a consequence, these strains are less effective, due to the complexity of the hydrolysate of lignocellulosic biomass as well as the small genome of the bacterium that must accommodate more and more genes to function properly. By contrast, genome-guided top-down strategies would provide more effective solutions for the challenge.

The complete genome of *Z. mobilis* ZM4 was sequenced in 2005 [35], and progress in its analysis and annotation has been made continuously since then [54,55]. This progress helps to elucidate the molecular basis of the unique physiological and metabolic traits observed in *Z. mobilis* such as the ED pathway. It also provides information for systems analysis of the bacterium's responses to ethanol and inhibitors, particularly at universal levels such as general stress response [56,57], so that top-down strategies can be developed for strain development through rational design, not only for more efficient cellulosic ethanol production [58] but also for the production of other bulk commodities [59–61].

Prospects for the Industrial Applications of *Z. mobilis*

Fuel ethanol from grains is produced at an ethanol titer as high as 12–14% (v/v) to save energy consumption in product recovery. Moreover, the stillage is treated by multievaporation for all residues left after ethanol distillation to be recovered and processed as animal feed to credit fuel ethanol production, and in the meantime condensate with small amounts of volatile byproducts is recycled. By contrast, cellulosic ethanol production cannot be performed in this mode, and the ethanol titer achieved so far has been 5–7% (v/v) only. Therefore, more stillage is discharged, and it must be treated properly. The most important aspect for commercial production of cellulosic ethanol is to reduce stillage discharge by increasing its ethanol titer.

SHCF with lignin separated before fermentation is highly recommended so that the fermentation can be operated under high-gravity conditions, but sugar loss with lignin cake presents a disadvantage. When *Z. mobilis* strains engineered with pentose metabolism are used, improved ethanol yield might compensate for the sugar loss. If these strains are further engineered with the self-flocculating phenotype or the self-flocculating *Z. mobilis* strains are engineered with pentose metabolism, biomass accumulated during ethanol fermentation can be recovered by sedimentation with negligible cost. Since *Z. mobilis* is generally regarded as a safe species with a protein content as high as 65%, much higher than that of *S. cerevisiae* [14], its biomass can be processed as animal feed or other value-added byproducts to credit cellulosic ethanol production. Without lignin and the biomass of *Z. mobilis*, the stillage is much cleaner and consequently suitable for recycling at a high ratio to reduce discharge.

So far, cellulosic ethanol has not been produced stably at a commercial scale. Despite many pilot and demonstration plants established worldwide, the former DuPont facility in Nevada, Iowa (USA) was the only one designed for cellulosic ethanol production from corn stover using the *Z. mobilis* strain jointly developed with NREL, with a projected production capacity of 30 million gallons of fuel ethanol per year [5]. It was opened in October 2015, but unfortunately closed in late 2017 due to poor economic performance. Therefore, additional pilot or demonstration plants are needed to study cellulosic ethanol production by *Z. mobilis* strains, especially to verify their advantages over strains of *S. cerevisiae*.

Concluding Remarks

Z. mobilis is not suitable for 1G fuel ethanol production, but it presents advantages for cellulosic ethanol production, because its ED pathway produces less ATP and biomass for more sugar to be used for ethanol production, and the bifurcated metabolic routes in the ED pathway partly decouple cell growth from ethanol production to succeed ethanol fermentation even without cell growth. However, improved ethanol yield may not be obtained if the biomass density of *Z. mobilis* is too low, and underlying fundamentals need to be elucidated.

No cofactor imbalance is observed for *Z. mobilis* engineered with pentose metabolism through the isomerase pathway. Since ethanol produced from lignocellulosic biomass cannot be as high as that produced from sugar and grains, tolerance of *Z. mobilis* to ethanol is sufficient for cellulosic ethanol production. Therefore, more effort should be devoted to exploring the mechanism underlying its tolerance to inhibitors released during the pretreatment of lignocellulosic biomass for engineering the bacterium to convert both C6 and C5 sugars in toxic hydrolysates into ethanol with high yield (see Outstanding Questions).

The self-flocculation of *Z. mobilis* is superior to its unicellular morphology. When the bacterial cells self-flocculate, they can be immobilized within fermenters under continuous fermentation conditions for high cell density to improve ethanol productivity. Meanwhile, their tolerance to inhibitors could be improved for more efficient production of cellulosic ethanol, but the molecular mechanisms underlying the self-flocculation of *Z. mobilis* and enhanced tolerance to inhibitors associated with the morphological change need to be explored for controlling the morphology properly (see Outstanding Questions).

Outstanding Questions

How can *Z. mobilis* be engineered for robust production of cellulosic ethanol through enhancing its tolerance to inhibitors released during the pretreatment of lignocellulosic biomass for more complete conversion of sugars in the hydrolysate to save feedstock consumption, and in the meantime reduce workload for stillage treatment?

How do the different inhibitors generated in the hydrolysate of lignocellulosic biomass poison the bacterial cells, separately as well as synergistically? What are the mechanisms underlying the tolerance of *Z. mobilis* to those inhibitors? Can we develop general strategies for stress response to major inhibitors instead of the current methodology, targeting individual inhibitors or categories, for an ultimate solution to this problem?

What is the mechanism underlying enhanced stress tolerance observed with the self-flocculating *Z. mobilis*? Can environmental stresses trigger QS with the bacterial flocs, similarly to other Gram-negative bacteria such as *E. coli* and *P. aeruginosa* under high cell-density conditions? Why no direct evidence, such as autoinducers or signal molecules that coordinate QS in other bacteria, has been reported for *Z. mobilis* so far?

What is the molecular mechanism underlying the self-flocculation of ZM401, and how does the deletion mutation of only one thymine of the nine consecutive thymines in the short sequence ZMO1082 upstream of ZMO1083 that encodes the catalytic subunit A of the **bacterial cellulose synthase** (BcsA) in *Z. mobilis*, make its cellulose biosynthesis significantly different with the formation of cellulose fibrils for developing such a unique phenotype, since the mutation destroys both the start code of ZMO1083 and the stop code of ZMO1082 for their integration to form a larger gene with new functions?

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