Overcoming the thermodynamic equilibrium of an isomerization reaction through oxidoreductive reactions for biotransformation

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Isomerases perform biotransformations without cofactors but often cause an undesirable mixture of substrate and product due to unfavorable thermodynamic equilibria. We demonstrate the feasibility of using an engineered yeast strain harboring oxidoreductase reactions to overcome the thermodynamic limit of an isomerization reaction. Specifically, a yeast strain capable of consuming lactose intracellularly is engineered to produce tagatose from lactose through three layers of manipulations. First, GAL1 coding for galactose kinase is deleted to eliminate galactose utilization. Second, heterologous xylose reductase (XR) and galactitol dehydrogenase (GDH) are introduced into the Δgal1 strain. Third, the expression levels of XR and GDH are adjusted to maximize tagatose production. The resulting engineered yeast produces 37.69 g/L of tagatose from lactose with a tagatose and galactose ratio of 9:1 in the reaction broth. These results suggest that in vivo oxidoreductase reactions can be employed to replace isomerases in vitro for biotransformation.
thermodynamic equilibrium where concentrations of substrates and products do not change with time is a fundamental limitation of enzymatic reactions catalyzed by isomerases. Biotransformations involving an isomerization reaction are therefore constrained by the underlying thermodynamic equilibrium, which often leads to an incomplete conversion of a substrate—generating a mixture of substrates and products. One typical example is the production of high-fructose corn syrup (HFCS) from glucose using glucose isomerase. Complete conversion of glucose into fructose is impossible due to the thermodynamic equilibrium of the reaction. Typically, a mixture of 42% fructose and 52% glucose is obtained by glucose isomerase. In order to increase the concentration of fructose, separation and enrichment of fructose from the mixture are necessary.

Due to increasing concerns about excessive calories from added sugars in food, consumers seek a low-calorie alternative. Rare sugars such as tagatose and allulose, which possess a sweetness profile similar to sucrose but with much fewer calories, are promising low-calorie sweeteners in the food industry. Rare sugar manufacturing usually involves isomerization reactions, resulting in a mixture of substrates and products after conversion. As such, commercial production of the rare sugars requires additional separation steps. Manufacturing costs of rare sugars tend to be higher than HFCS and have impeded the wide use of rare sugars, even though they exhibit many benefits especially as potential anti-diabetic and anti-obesity medicines. In particular, tagatose, a naturally occurring functional sweetener with 92% of the sweetness of sucrose but with substantially fewer calories (1.5–2.5 kcal/g vs. 4 kcal/g sucrose) has drawn great attention. Tagatose produced through isomerization of galactose is used as a food ingredient and attained GRAS (generally recognized as safe) status under the U.S. Food and Drug Administration (FDA). However, the manufacturing cost of tagatose by enzymatic isomerization remains high, partly due to the unfavorable thermodynamic equilibrium between galactose and tagatose. Although the ratio of galactose and tagatose after enzymatic isomerization can be shifted to 4:6 by increasing reaction temperatures to 60 °C, the ratio of galactose and tagatose is around 7:3 under the temperatures, where L-arabinose isomerase (L-AI) is active and stable. To obtain galactose as a substrate, enzymatic hydrolysis of lactose followed by a separation of glucose and galactose is required, and these additional steps can increase production costs. Moreover, the long-term and large-scale enzymatic conversion processes require maintenance costs, such as continued production of expensive purified enzymes. Therefore, enzyme-based tagatose production cannot compete with HFCS in the market despite its health benefits.

To bypass the above-mentioned bottlenecks, we devise a scalable and cost-effective tagatose production strategy based on direct yeast fermentation of lactose, an abundant sugar found in dairy by-products. Specifically, we develop a whole-cell conversion process where lactose is transported into the cytosol of yeast, hydrolyzed, and converted into tagatose. In order to overcome the unfavorable equilibrium (7:3) by an isomerase reaction between galactose and tagatose at 30 °C, a two-step oxido-reductive pathway, consisting of aldose reductase and galactitol-2-dehydrogenase, is introduced into an engineered Saccharomyces cerevisiae capable of assimilating lactose. Additionally, to enhance the self-sustained production of tagatose in the lactose-assimilating yeast with the oxido-reductive pathway, galactose kinase (Gal1) is inactivated. As such, the resulting engineered yeast can take up lactose, produce glucose and galactose intracellularly. The intracellular glucose is used as the energy source for cell growth and maintenance, while galactose is simultaneously converted into tagatose. In this study, we demonstrate efficient tagatose production from lactose by the engineered yeast strain through a carbon-partition strategy whereby a consumed disaccharide is rerouted into different metabolic pathways—one for cell growth and maintenance, and another for targeted product generation. Interestingly, the production of tagatose directly from lactose by engineered yeast via the oxido-reductive pathway leads to a favorable conversion ratio (1.9) of galactose and tagatose, exceeding the thermodynamic equilibrium (7:3) by an isomerization reaction at 30 °C.

Conversion of galactose into tagatose by oxido-reductases. To overcome the unfavorable equilibrium between galactose and tagatose by an isomerization reaction, we employed two oxido-reductases commonly used for the isomerization of aldose and ketose in yeast and fungi for the intracellular conversion of galactose into tagatose. As xylose reductase (XR) is known to reduce galactose into galactitol and Scheffersomyces stipitis XYL1 coding for NADPH-linked XR was expressed in the EJ2g strain to produce galactitol from lactose. Expressing XR using a multicopy plasmid (pRS42K) and a strong constitutive promoter (PTDH) (EJ2g_pX) resulted in the production of 3.5 g/L of galactitol during lactose fermentation (Fig. 2 and Supplementary Figure 2). To convert galactitol into tagatose, an NAD+–linked galactitol-2-dehydrogenase (GDH) from Rhizobium leguminosarum was expressed in the galactitol-producing strain (EJ2g_pX) using a strong promoter (PTDH) and a multicopy plasmid (pRS42H), resulting in the EJ2g_pXP strain. The EJ2g_pXP strain produced 11.43 g/L of tagatose from 47.2 g/L of lactose (Fig. 3 and Supplementary Figure 4). These data demonstrated that the two oxido-reductases (XR and GDH) functionally expressed in the engineered yeast (EJ2g_pXP) and they enabled direct production of tagatose from lactose.

Fine-tuning of XR and GDH for enhanced tagatose production. While the engineered yeast EJ2g_pXP produced tagatose from lactose, the observed yield (0.242 g tagatose/g lactose) was only half of the theoretical yield (0.526 g tagatose/g lactose). We reasoned that the XR and GDH expression levels might need optimization to minimize the formation of by-products, such as galactose and galactitol (Fig. 3b, c), and maximize tagatose production. To elucidate the relationships between the galactose, galactitol, and tagatose production levels and the expression levels of XR and GDH, we constructed three more engineered strains with additional copies of XR and GDH via Cas9-based genome integration. The production of galactose, galactitol, and...
Fig. 1 Galactose accumulation in lactose-consuming strain EJ2 with GAL1 deletion (EJ2g). a Growth profile as shown by dry cell weight (DCW), b lactose consumption, c ethanol production, and d galactose production by the EJ2g strain (filled circles) as compared with the EJ2 control strain (open circles) under aerobic conditions. Data are presented as mean value and standard deviations of three independent biological replicates. Source data are provided as a Source Data file.

Fig. 2 Galactitol production after xylose reductase (XR) introduction into EJ2g. a Growth profile as shown by dry cell weight (DCW), b lactose consumption, c galactose production, and d galactitol production by the EJ2g strain with XR overexpression in the pRS42K plasmid (EJ2g_pX, filled circles), and EJ2g with empty plasmid pRS42K as control (open circles) on YP medium with 40 g/L lactose under aerobic conditions. Data presented as mean values and standard deviations of three independent biological replicates. Source data are provided as a Source Data file.
tagatose from lactose by the four engineered yeast strains EJ2g_pXpG, EJ2g_iXiG_pX, EJ2g_iXiG_pG, and EJ2g_iXiG_pXpG (Table 1) was measured by HPLC (Fig. 4 and Supplementary Figure 5). Additional overexpression of XR in the EJ2g_iXiG_pX strain resulted in increased galactitol yield, but the yield of tagatose was unchanged. By contrast, additional overexpression of GDH in the EJ2g_iXiG_pG strain led to less galactitol accumulation and more tagatose production than the parental strain EJ2g_iXiG. The EJ2g_iXiG_pXpG strain with overexpression of both XR and GDH exhibited the highest tagatose and lowest galactitol yields (Fig. 4). These results suggested that XR and GDH expression levels played a critical role in manipulating the metabolic fluxes toward tagatose production. Using simple modifications in the expression levels of XR and GDH, we increased the yield of tagatose from lactose by more than 20%. The final galactose to tagatose ratio of EJ2g_iXiG_pXpG strain in the fermentation broth was 2:8.

**Tagatose production in a bioreactor.** To increase the titer of tagatose and to investigate the feasibility of large-scale production of tagatose from lactose, the EJ2g_iXiG_pXpG strain exhibiting the highest tagatose titer from a shake flask fermentation was tested in a bioreactor (Fig. 5 and Supplementary Figure 6). After 50 g/L of lactose was consumed, additional feedings of lactose were conducted to maintain lactose concentrations around 10–20 g/L. After the fed-batch fermentation, 114.21 g/L of lactose was consumed and the titers of tagatose, galactose, and galactitol were 37.69 g/L, 4.41 g/L, and 8.46 g/L, respectively. As such, the conversion ratios of tagatose, galactose, and galactitol were 74.5%, 8.7%, and 16.7%, respectively. The tagatose yield from lactose was 0.33 g tagatose/g lactose, which is equivalent to 62.7% of a theoretical maximum (0.526 g tagatose/g lactose). Furthermore, the ratio of galactose to tagatose in the fermentation broth reached as high as 1:9 which is much higher than the ratio of 2:8 from the shake flask fermentation, substantially improving the 7:3 ratio achieved with the isomerase pathway at 30 °C. The concentration of galactose reached a steady-state level during the lactose feeding process (Fig. 5). The yield of galactitol in the fed-batch fermentation (0.074 g galactitol/g lactose vs. 0.035 g galactitol/g lactose) was higher than that in the shake flask fermentation, suggesting that GDH activity and supply of NAD⁺ might need to be further optimized to cope with higher metabolic fluxes from lactose and galactose.

![Fig. 3 Tagatose production after the introduction of galactitol-2-dehydrogenase (GDH).](https://example.com/f3.png)

**Fig. 3** Tagatose production after the introduction of galactitol-2-dehydrogenase (GDH). a Growth curve as shown by DCW, b lactose consumption, c galactose production, and d galactitol production, and e tagatose production by the EJ2g_pXpG strain (filled circles) and the EJ2g_pX strain with p42H empty plasmid as control (open circles) under aerobic condition. Data are presented as mean value and standard deviations of three independent biological replicates. Source data are provided as a Source Data file.

### Table 1 Engineered *S. cerevisiae* strains used in this study

| Strains   | Description                                                                 | Source                      |
|-----------|-----------------------------------------------------------------------------|-----------------------------|
| EJ2       | Evolved strain of EJ1 (D452-2 leu2:LEU2 pRS405-gh1-1 ura3:URA3 pRS406-cdt-1) | *22*                        |
| EJ2g      | EJ2 Δgal1                                                                   | This study                  |
| EJ2g_pX   | EJ2g w/p42K-XR                                                              | This study                  |
| EJ2g_pXpG | EJ2g w/p42K-XR and p42H-GDH                                                  | This study                  |
| EJ2g_iX   | EJ2g w/integrated pTDH3-XYL1-tTDH3                                           | This study                  |
| EJ2g_iG   | EJ2g w/integrated pTDH3-GDH-tCYC1                                           | This study                  |
| EJ2g_iXiG | EJ2g w/integrated pTDH3-XYL1-tTDH3 and pTDH3-GDH-tCYC1                      | This study                  |
| EJ2g_iXiG_pX | EJ2g_iXiG w/p42K-XR                                                        | This study                  |
| EJ2g_iXiG_pG | EJ2g_iXiG w/p42H-GDH                                                          | This study                  |
| EJ2g_iX_pXpG | EJ2g_iX w/p42K-XR and p42H-GDH                                                | This study                  |
| EJ2g_iG_pXpG | EJ2g_iG w/p42K-XR and p42H-GDH                                                | This study                  |

**Table 1 Engineered *S. cerevisiae* strains used in this study**

- **Strains**: Evolved strain of EJ1, Δgal1, w/p42K-XR, with integrated pTDH3-XYL1-tTDH3, w/integrated pTDH3-GDH-tCYC1, w/p42K-XR and p42H-GDH, w/p42K-XR and p42H-GDH, w/p42K-XR and p42H-GDH, w/p42K-XR and p42H-GDH, w/p42K-XR, and p42H-GDH.
- **Description**: Evolved strain of EJ1 (D452-2 leu2:LEU2 pRS405-gh1-1 ura3:URA3 pRS406-cdt-1), Δgal1, w/p42K-XR, with integrated pTDH3-XYL1-tTDH3, w/integrated pTDH3-GDH-tCYC1, w/p42K-XR and p42H-GDH, w/p42K-XR and p42H-GDH, w/p42K-XR and p42H-GDH, w/p42K-XR and p42H-GDH, w/p42K-XR, and p42H-GDH.
- **Source**: This study.
Discussion

In this study, we bypassed the thermodynamic equilibrium of the isomerase reaction by using an oxidoreductive pathway to achieve the one-pot biosynthesis (Fig. 6). Tagatose production was selected as a demonstration system as its production was shown previously to be limited by the thermodynamic equilibrium8,9,18,26–28.

Interconversion between aldose and ketose forms of sugars are critical for their assimilation by microorganisms. Both single-step isomerization and two-step oxidoreductive reactions have evolved to catalyze these interconversions. For instance, xylose can be converted into xylulose via either xylose isomerase (XI) or the combined action of xylose reductase (XR) and xylitol dehydrogenase (XDH)29. The XI reaction is often associated with anaerobic or microaerophilic bacteria30,31, due to the fact that the isomerase enzyme does not require cofactors and can carry out bioconversions under oxygen-limited and anaerobic conditions. Isomerase reactions have been adopted for industrial production of various value-added chemicals, but face two fundamental drawbacks in their use. First, the inherent thermodynamic
equilibrium between the substrate and product leads to low conversion yields and creates difficulties for downstream products separation and purification. Second, the scale-up cost sharply increases, because the amount of enzyme required is directly proportional to the proposed reaction scale. For example, rare sugars such as tagatose and allulose are currently produced by enzymatic reactions followed by complicated separation processes. As a result, the production costs of rare sugars are significantly higher than HFCS, which does not require additional separation. Due to this high production cost, introduction of rare sugars into foods and beverages has been hampered in spite of potential benefits of rare sugars.

Unlike the isomerase reaction, the oxidoreductive reaction consists of two steps (an oxidation and a reduction) and requires cofactors. Though seemingly redundant, this mechanism prevents the undesired reverse reaction through thermodynamic coupling to cofactor equilibria. Therefore, we used the oxidoreductive pathway instead of the isomerase pathway, to achieve more complete conversion of galactose into tagatose product than the isomerization. To implement the oxidoreductive pathway for tagatose production, in vivo bioconversion is more suitable than in vitro enzymatic conversion, considering the need for redox cofactors. Simultaneously, an efficient self-sustained bioconversion system is advantageous when considering scale-up costs. While direct and efficient conversion of lactose into tagatose by the oxidoreductive pathway has been demonstrated in this study, the secretion of galactose and galactitol during the conversion needs to be addressed in order to reach the theoretical maximum yield. We speculate that the secretion of galactose and galactitol might be facilitated by endogenous hexose and sugar alcohol transporter in yeast. Specifically, Gal2 (galactose permease) might be responsible for the secretion and re-assimilation of galactose, and Fps1 (aquaglyceroporin), which have been reported to transport xylitol, might be involved in the secretion of galactitol. From the structural similarity between galactitol and xylitol, the deletion of FPS1 in a tagatose-producing strain might reduce production of galactitol. As Gal2 might be involved in both secretion and re-assimilation of galactose, upregulation of GAL2 might increase the secretion and re-assimilation of galactose, and deletion might reduce the secretion and re-assimilation of galactose. As such, the effects of GAL2 perturbation would likely be mixed or compromised. Genetic perturbations of endogenous sugar and sugar alcohol transporters including FPS1 and GAL2 can be conducted in the future to further improve the conversion yield of tagatose from lactose. In addition to galactitol accumulation as a by-product, the productivity and titer of tagatose from lactose by our engineered yeast need to be further improved. While the productivity (0.126 g/L/h) and titer (37.69 g/L) of tagatose production from lactose by our engineered yeast are comparable with the reported productivities (0.103–0.896 g/L/h) and titers (14.8–21.5 g/L) of enzyme-based tagatose production from lactose, they are much lower than the productivity (7.9 g/L/h) and titer (158 g/L) by enzyme-based tagatose production from galactose. Continuous fermentation with cell-recycling might be a possible approach to improve the productivity of tagatose production from lactose by our engineered yeast.

We also designed a carbon partition strategy that utilizes lactose, a disaccharide of galactose, and glucose, as an initial substrate for simultaneous tagatose production and glucose catabolism. Specifically, we intentionally shut down the native galactose utilization pathway of S. cerevisiae and redirected galactose toward tagatose through the introduced oxidoreductive pathway. Meanwhile, glucose was consumed to sustain the engineered yeast via its native pathway. Because the two monosaccharides were released intracellularly, the glucose repression on galactose uptake was bypassed and thus allowed for simultaneous utilization. Hydrolysis of lactose into glucose and galactose, and the subsequent conversion of galactose to tagatose, were integrated and self-sustained, which dramatically reduced processing costs. It is worth noting that the carbon-partition strategy is not only limited to tagatose production but can also be applied in other bioconversion systems. When consuming disaccharides, we can opt to turn off the native catabolic pathway of one of the monosaccharide moieties and reprogram this monosaccharide toward our target chemicals, while leaving another moiety for cell growth and maintenance. If it is simpler to produce the target chemical using glucose rather than galactose as a substrate, we can turn off the glucose pathway by disrupting the hexose kinases (encoded by HXK1 and HXK2) and glucose kinase (encoded by GLK1). We can then introduce a target oxidoreductive pathway to allow glucose rerouting to the target chemicals, while maintaining the native galactose pathway for cell maintenance. Apart from lactose, other disaccharides such as sucrose are also cheap and abundant. Many other engineered yeast strains rapidly consume these disaccharides as well. Thus, we can produce target chemicals from these disaccharides as needed through the carbon-partition strategy.

In this study, our strategy reduces the production cost at every step, as compared with existing industrial tagatose production systems. First, the traditional process requires that the majority of galactose is made from enzymatic hydrolysis of lactose, followed by the separation of glucose and galactose. Therefore, direct consumption of lactose by our engineered yeast strain can significantly reduce the enzyme (β-galactosidase) and separation costs. In addition, lactose is readily obtained from whey, an abundant waste product from cheese and Greek yogurt production. Next, the in vivo oxidoreductive conversion of galactose into tagatose eliminated the cost of purified or immobilized L-arabinose isomerase. Unlike the traditional process, where enzyme cost increases proportionally with the scale of operation, the engineered yeast replicates itself continuously regardless of the reaction scale. Most importantly, the oxidoreductive reaction allows near-complete conversion of galactose into tagatose. The small amounts of galactose in the fermentation broth can be completely removed by adding regular yeast that consumes galactose to simplify the downstream separation process. Therefore, our strategy not only maximizes substrate value, but also simplifies product separation and purification, resulting in an overall decrease in production costs. In future studies, we intend to optimize the oxidoreductive galactose–tagatose pathway, to increase tagatose yields, to achieve complete conversion of galactose into tagatose, and to further minimize the separation and purification cost. In summary, we envision the carbon-partition strategy applicable for value-added chemical productions from disaccharides by engineered microorganisms. Our scheme allows for the disaccharides to be utilized intracellularly, and its monosaccharide products allotted for maintenance and production separately, but simultaneously.

**Methods**

**Strains and media.** *Escherichia coli* Top10 (Invitrogen, Grand Island, NY) was used for the construction and propagation of plasmids. *E. coli* was grown in the Luria-Bertani medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.0) at 37 °C, and ampicillin (100 μg/mL) was added for selection when required. Yeast strains were grown on theYPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) at 30 °C. Yeast strains transformed with plasmids containing antibiotic markers were propagated on YPD plates supplemented with the corresponding antibiotics, such as hygromycin B (300 μg/mL) and/or geneticin G418 (300 μg/mL).

**Plasmids and strain construction.** The guide RNA plasmid p42H-gGAL1 (Supplementary Table 3) was amplified from gRNA-ura-HYB25,36 used as a template by
using primer pair Gal1-gU and Gal1-gD (Supplementary Table 1) carrying a 20 bp PAM sequence for using Cas9-based genome integration. The transformants were confirmed by PCR using primers CS6-CKU and CS6-CKD, and was designated as the EJ2g_iXiG strain (Table 1). For genomic integration of YXL1 into the EJ2g strain, the plasmid p42K-CSW was amplified using a primer pair of CSW-1 and CSW-2 and digested with Sall and Kpn1, ligated with pRS42K and Sall and Kpn1 digested p42H-GDH, forming plasmid p42HG-pDHG (Supplementary Table 3). The genome of DHG was transformed into gBlocks (IDT Inc., Skokie, IL) and blunt-ligated with plasmid p42H-gPDP to generate plasmid p42H-GDH.

### Yeast culture and fermentation conditions

The lactose fermentation was started by inoculating the overnight yeast pre-culture (5 mL of the YP medium containing 20 g/L of lactose) into 20 mL YPL (the YP medium containing 40 g/L of lactose with hygromycin (300 μg/mL) and抗生素 was slowly fed through a pump to maintain the lactose concentration at 10–20 g/L. The feeding rate was manually adjusted based on lactose consumption and lactose concentration in the fermenter. The culture pH was automatically maintained at 5.6 by adding 3 N NaOH or HCl solution. The gas flow rate was set at 1vvm, and agitation was set at 800 rpm.

### Analytical methods and metabolite analysis

The ODH of cultures was measured using a spectrophotometer (BioMate 5, Thermo, NY) and dried cell weight (DCW) was obtained from an experimentally determined conversion factor of 0.454 g DCW/OH. Extracellular metabolites such as lactose, galactose, galactose, glycerol, acetate, and ethanol were measured by HPLC (Agilent Technologies 1200 Series, Santa Clara, CA) with a Rezex<sup>®</sup> ROA-Acidic H+ column (8% column (Phenomenex Inc., Torrance, CA) and a refractive index detector (RID). The column was eluted with 0.005 N H2SO4 at a flow rate of 0.6 mL/min at 50 °C. Galactose and tagatose cannot be separated by this ROA-acidic H+ column (Supplementary Table 3). Galactose and tagatose concentrations were measured using an Agilent Technologies 1200 Series HPLC equipped with a Rezex<sup>®</sup> RCM-MonoSacharide CA + 2 (8% column (Phenomenex Inc. Torrance, CA) and RID detector). The mobile phase was composed of water (Barnstead E-Pure<sup>®</sup>) Water Purification Systems, Thermo Scientific) and was eluted at a flow rate of 0.6 mL/min at 80 °C. The maximum theoretical tagatose yield (0.501 g/g lactose) by engineered yeast was calculated based on the molecular weights of tagatose (180.16 g/mol) and lactose (342.3 g/mol) with an assumption that glucose is not converted into tagatose.

### Data availability

Data supporting the findings of this work are available within the paper and its Supplementary Information files. A reporting summary for this article is available as a Supplementary Information file. The source data underlying Figs. 1, 2, 3, 4, and 5 are provided as a Source Data file. All other data are available from the corresponding author upon reasonable request.

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Author contributions
Y.-S.J., J.-J.L. and G.-C.Z. designed research. J.-J.L. and G.-C.Z. performed research and analyzed data. S.K. and E.J.O. helped with bioreactor fermentation. J.-J.L., G.-C.Z., E.J.Y., K.C., J. H.D.C., and Y.-S.J. wrote the paper. All authors read and approved the paper.

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Competing interests: J.-J.L., G.-C.Z., K.C., J.H.D.C. and Y.-S.J. are authors of a patent application filed by University of Illinois at Urbana-Champaign based on this work. K.C., J.H.D.C. and Y.-S.J. have financial interest in Sugarlogix, Inc. The remaining authors declare no competing interests.

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