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PHYSIOLOGY

A Mutation in *PGM2* Causing Inefficient Galactose Metabolism in the Probiotic Yeast *Saccharomyces boulardii*

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ABSTRACT The probiotic yeast *Saccharomyces boulardii* has been extensively studied for the prevention and treatment of diarrheal diseases, and it is now commercially available in some countries. *S. boulardii* displays notable phenotypic characteristics, such as a high optimal growth temperature, high tolerance against acidic conditions, and the inability to form ascospores, which differentiate *S. boulardii* from *Saccharomyces cerevisiae*. The majority of prior studies stated that *S. boulardii* exhibits sluggish or halted galactose utilization. Nonetheless, the molecular mechanisms underlying inefficient galactose uptake have yet to be elucidated. When the galactose utilization of a widely used *S. boulardii* strain, ATCC MYA-796, was examined under various culture conditions, the *S. boulardii* strain could consume galactose, but at a much lower rate than that of *S. cerevisiae*. While all *GAL* genes were present in the *S. boulardii* genome, according to analysis of genomic sequencing data in a previous study, a point mutation (G1278A) in *PGM2*, which codes for phosphoglucomutase, was identified in the genome of the *S. boulardii* strain. As the point mutation resulted in the truncation of the Pgm2 protein, which is known to play a pivotal role in galactose utilization, we hypothesized that the truncated Pgm2 might be associated with inefficient galactose metabolism. Indeed, complementation of *S. cerevisiae PGM2* in *S. boulardii* restored galactose utilization. After reverting the point mutation to a full-length *PGM2* in *S. boulardii* by Cas9-based genome editing, the growth rates of wild-type (with a truncated *PGM2* gene) and mutant (with a full-length *PGM2*) strains with glucose or galactose as the carbon source were examined. As expected, the mutant (with a full-length *PGM2*) was able to ferment galactose faster than the wild-type strain. Interestingly, the mutant showed a lower growth rate than that of the wild-type strain on glucose at 37°C. Also, the wild-type strain was enriched in the mixed culture of wild-type and mutant strains on glucose at 37°C, suggesting that the truncated *PGM2* might offer better growth on glucose at a higher temperature in return for inefficient galactose utilization. Our results suggest that the point mutation in *PGM2* might be involved in multiple phenotypes with different effects.

IMPORTANCE *Saccharomyces boulardii* is a probiotic yeast strain capable of preventing and treating diarrheal diseases. However, the genetics and metabolism of this yeast are largely unexplored. In particular, molecular mechanisms underlying the inefficient galactose metabolism of *S. boulardii* remain unknown. Our study reports that a point mutation in *PGM2*, which codes for phosphoglucomutase, is responsible for inferior galactose utilization by *S. boulardii*. After correction of the mutated *PGM2* via genome editing, the resulting strain was able to use galactose faster than a parental strain. While the *PGM2* mutation made the yeast use galactose slowly, investigation of the genomic sequencing data of other *S. boulardii* strains revealed that the

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PGM2 mutation is evolutionarily conserved. Interestingly, the *PGM2* mutation was beneficial for growth at a higher temperature on glucose. We speculate that the *PGM2* mutation was enriched due to selection of *S. boulardii* in the natural habitat (sugar-rich fruits in tropical areas).

KEYWORDS *Saccharomyces boulardii*, galactose, *PGM2*, genome editing

Saccharomyces boulardii was first isolated from the surfaces of mangosteen and lychee fruits, and this yeast has been proven to be an effective dietary supplement for treating diarrhea (1, 2). *S. boulardii* has been extensively studied for its probiotic effects against gastrointestinal tract disorders (3–14). The clinical activities of *S. boulardii* relevant to antibiotic-associated diarrhea and recurrent *Clostridium difficile* intestinal infections have been reported (8, 15–18). *S. boulardii* is also called *S. cerevisiae* var. *boulardii*, and it belongs to the same species as *Saccharomyces cerevisiae* (2, 9, 19). However, *S. boulardii* exhibits unique characteristics that make this yeast different from *S. cerevisiae*. Specifically, *S. boulardii* has a trisomy of chromosome IX, as well as higher copy numbers of particular sets of genes (including some stress-responsive genes) and fewer delta (δ) transposon sequences than *S. cerevisiae* (9, 20, 21). Notably, *S. boulardii* is able to tolerate and grow at a mammalian internal temperature of 37°C and under acidic conditions in the stomach, which makes this yeast capable of staying longer in the human gut as a probiotic (22).

In addition to the differences between *S. cerevisiae* and *S. boulardii* mentioned above, there have been conflicting reports regarding the galactose utilization phenotypes of *S. boulardii*. Although it was reported, based on the genomic sequencing data of *S. boulardii*, that *S. boulardii* has all of the genes in its genome necessary for galactose uptake and utilization (23) (Fig. 1), galactose utilization by *S. boulardii* remained controversial. In 1996, it was reported that *S. boulardii* was a wild *Saccharomyces* strain that does not utilize galactose as a carbon source (24). However, detailed genetic or biochemical characterizations supporting this conclusion were not provided in the report. Two years later, McCullough et al. stated that three *S. boulardii* strains used in their study were able to ferment galactose, but this "fermentation" was qualitatively examined only on solid agar plates (19). In 2001, Mitterdorfer et al. claimed, based on automated turbidimetric measurements, that none of the so-called *S. boulardii* strains they tested could utilize galactose (25). One strain isolated from Enterol was claimed to be capable of assimilating galactose, based on analysis using the 20 C AUX kits (API) (26). Taken together, these findings mean that the galactose fermentation capability of *S. boulardii* remains unclear because the conclusions from the prior studies were derived from insufficient data, using different means of measurements, and under different culture conditions. Therefore, a detailed and comprehensive investigation of the galactose utilization phenotype of *S. boulardii* is necessary. In this study, we performed yeast fermentations of a widely used *S. boulardii* strain, ATCC MYA-796, under different fermentation conditions and using galactose as a sole carbon source. The results showed that the *S. boulardii* strain could assimilate galactose but at a much lower rate than that of *S. cerevisiae* BY4742. We confirmed that this lower galactose utilization by *S. boulardii* was attributed to a single point mutation, G1278A, in *PGM2*. Interestingly, other recently sequenced *S. boulardii* strains contained the same point mutation in *PGM2*, suggesting that there might be physiological benefits from this mutation. We performed detailed comparisons of wild-type *S. boulardii* and *S. boulardii* with a functional Pgm2, constructed by CRISPR-Cas9 technology. Our results indicated that a wild-type *S. boulardii* strain with a defective Pgm2 possessed a growth advantage when grown on glucose under higher temperatures.

RESULTS

***S. boulardii* is capable of utilizing galactose.** Galactose fermentation experiments were conducted to examine the galactose utilization capability of the *S. boulardii* strain ATCC MYA-796, which has been widely used and genome sequenced. As shown in Fig. 2, *S. boulardii* MYA-796, here *S. boulardii*, consumed 50 g/liter of galactose over

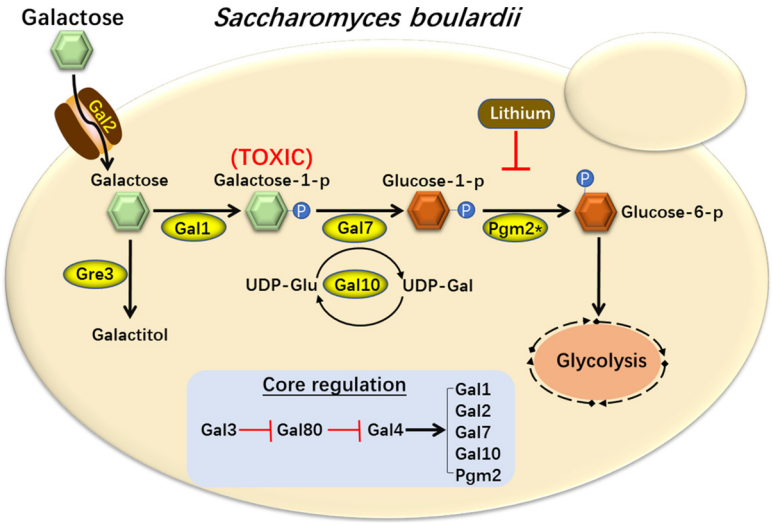

FIG 1 Schematic overview of galactose utilization and Pgm2 inactivation-induced galactose toxicity in *S. boulardii*. Gal2, galactose permease; Gal1, galactokinase; Gre3, aldose reductase; Gal7, galactose-1-phosphate uridyl transferase; Gal10, UDP-glucose-4-epimerase; Gal3, transcriptional regulator; Gal80, transcriptional repressor; Gal4, transcriptional activator; Pgm2, phosphoglucomutase; galactose-1-p, galactose-1-phosphate; glucose-1-p, glucose-1-phosphate; glucose-6-p, glucose-6-phosphate; UDP-Glu, UDP glucose; UDP-Gal, UDP galactose.

48 h, while *S. cerevisiae* strain BY4742 consumed the same amount of galactose within 24 h (Fig. 2A and B). These results indicated that *S. boulardii* could consume galactose but that the rate of galactose consumption by *S. boulardii* was much lower than that by *S. cerevisiae*. Previous studies reported that galactose consumption (even by *S. cerevisiae*) was influenced by oxygen levels, and most yeasts exhibit poor galactose fermentation under strictly anaerobic conditions in general (27–29). Therefore, galactose utilization rates by *S. boulardii* and *S. cerevisiae* were examined under different aeration levels (see Fig. S1 in the supplemental material). Consistent with observations from previous reports, both *S. boulardii* and *S. cerevisiae* exhibited higher galactose utilization rates under aerobic conditions than under anaerobic conditions. The galactose utiliza-

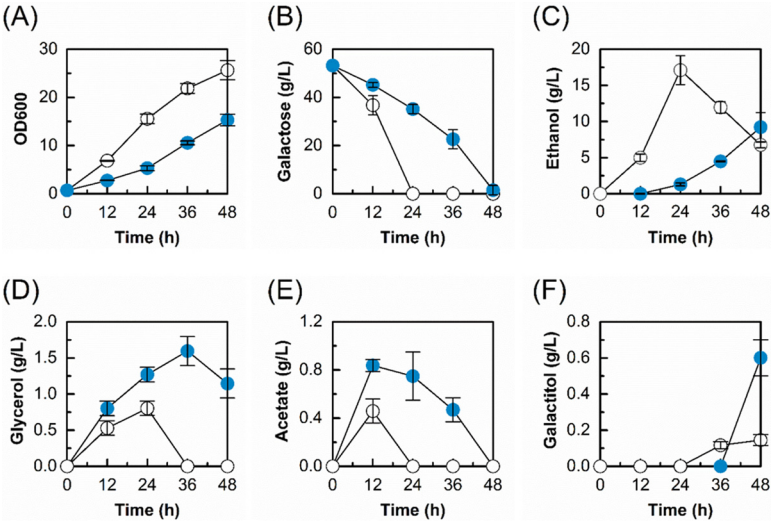

FIG 2 *S. boulardii* (ATCC MYA-796) is able to slowly consume galactose. The growth (A), galactose consumption (B), and ethanol production (C) of *S. boulardii* (closed circle) and *S. cerevisiae* BY4742 (open circle) cultures on galactose under aerobic conditions. *S. boulardii* produced larger amounts of glycerol (D), acetate (E), and galactitol (F) than did *S. cerevisiae* BY4742. Results are the means of duplicate experiments, and error bars indicate standard deviations.

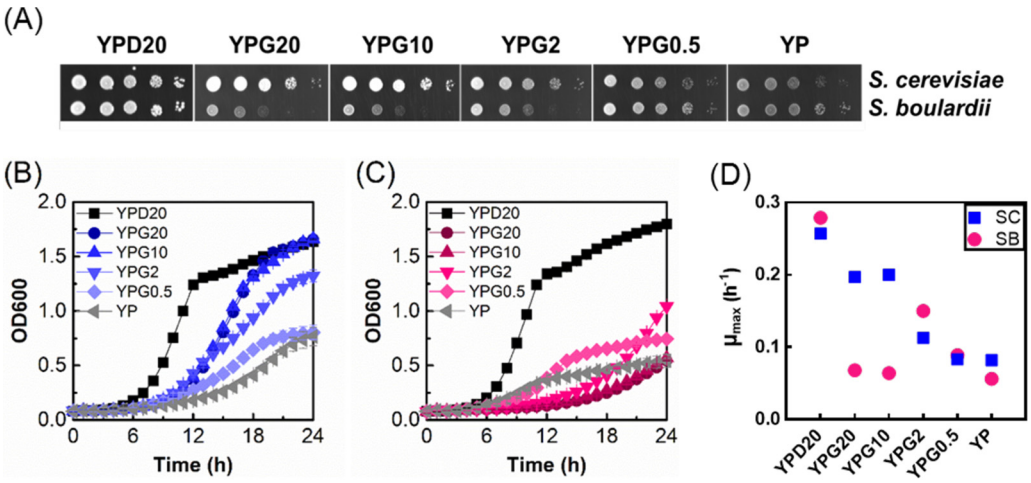

FIG 3 Galactose is toxic to *S. boulardii*. (A) Serial dilution of *S. boulardii* and *S. cerevisiae* onto YP plates with different galactose concentrations (20 g/liter, 10 g/liter, 2 g/liter, and 0.5 g/liter) using YPD20 (YP plates with 20 g/liter of glucose as carbon source) and YP without any sugar as the control. Yeast cells were grown to the stationary phase in YPD liquid medium and 1 OD_{600} unit/ml of yeast cells was collected and washed twice. One OD_{600} unit per milliliter of yeast cells was 10-fold serially diluted and plated as indicated. The plates were then incubated at 30°C for 36 h prior to pictures being taken. (B) The growth of *S. cerevisiae* BY4742 in YP medium with different galactose concentrations, as monitored by a Bioscreen C plate reader. (C) The growth of *S. boulardii* in YP medium with different galactose concentrations measured by using a Bioscreen C reader. (D) The correlation of the maximum specific growth rates of *S. cerevisiae* BY4742 (SC) and *S. boulardii* (SB) with different galactose concentrations. Results are the means of triplicate experiments; error bars indicate standard deviations and are not visible when smaller than the symbol size.

tion rate by *S. boulardii* under oxygen-limited conditions was much lower than that of *S. cerevisiae*, suggesting that galactose metabolism by *S. boulardii* might be more dependent on aeration than that by *S. cerevisiae*. It was reported that the optimal growth temperature of *S. boulardii* on glucose was 37°C rather than 30°C (30, 31). We also checked the growth temperatures of *S. boulardii* under galactose conditions. The results indicated that *S. boulardii* grew better on galactose at 30°C than at 37°C (Fig. S1).

Low galactose consumption by *S. boulardii* is related to galactose toxicity. *S. boulardii* consumed galactose at a lower rate than *S. cerevisiae* BY4742, regardless of growth temperature and aeration. Notably, the concentrations of glycerol, acetate, and galactitol were much higher in the culture media of *S. boulardii* than in those of *S. cerevisiae* (Fig. 2D to F), indicating that galactose metabolism in *S. boulardii* might not be streamlined as well as in *S. cerevisiae*. In relation to this observation, it was reported that *S. cerevisiae* could produce more glycerol and acetate on galactose medium in the presence of lithium ions (32), because lithium ions inhibit phosphoglucomutase (encoded by *PGM2*) and cause galactose toxicity (33) (Fig. 1). We therefore hypothesized that slow and inefficient galactose metabolism of *S. boulardii* might be caused by a similar mechanism. To investigate this phenomenon, serial dilutions and platings of *S. boulardii* and *S. cerevisiae* onto agar plates with different concentrations of galactose as a sole carbon source were performed (Fig. 3A; see also Fig. S2 in the supplemental material). *S. cerevisiae* colonies grew well, regardless of galactose concentrations. However, *S. boulardii* colonies showed a growth defect on the plates with higher concentrations of galactose. The growth defect of *S. boulardii* colonies under high galactose concentrations was also confirmed by liquid cultures with differential galactose concentrations using a Bioscreen C plate reader (Fig. 3B to D). These results indicated that galactose at high concentrations is toxic to *S. boulardii* and that the toxicity is galactose dose dependent.

Introduction of the *S. cerevisiae PGM2* gene enhanced the galactose utilization of *S. boulardii*. As the growth defect of *S. boulardii* colonies on galactose is similar to that of *S. cerevisiae* colonies associated with lithium-induced galactose toxicity (34), we reasoned that the growth defect of *S. boulardii* and galactose toxicity under high galactose concentrations might be related to a low activity of Pgm2. To confirm this

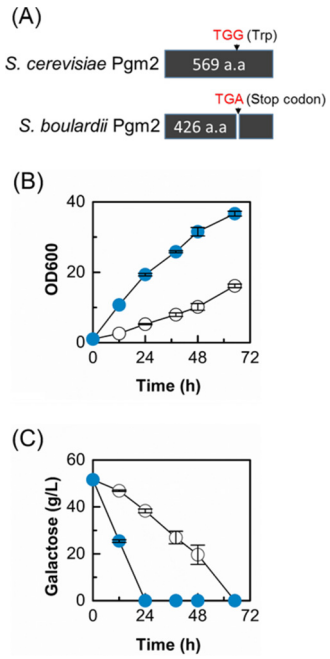

FIG 4 Introduction of *PGM2* restored galactose utilization in *S. boulardii*. (A) The point mutation G1278A in *PGM2* in *S. boulardii* led to a Pgm2 protein containing 426 amino acids, shorter that that of *S. cerevisiae* (569 amino acids). (B and C) The growth (B) and galactose consumption (C) of *S. boulardii* (open circle) and *S. boulardii* with *PGM2* overexpression (closed circle) on galactose under aerobic conditions. Results are the means of duplicate experiments, and error bars indicate standard deviations.

hypothesis, we first checked whether or not there are any mutations in *S. boulardii PGM2* using the genome sequence data (35). There are indeed three point mutations in the *S. boulardii PGM2* as compared to *S. cerevisiae PGM2*. Two point mutations (C267T and T1125C) are synonymous mutations, but the third mutation, G1278A (Trp codon to a stop codon), will lead to an early termination of translation (Fig. 4A). The early termination will form a truncated Pgm2 (426 amino acids instead of 569 amino acids), which might be a defective enzyme, as predicted by the Phyre2 Web portal (36) (see Fig. S3 in the supplemental material). We reasoned that the truncated Pgm2 protein might cause the growth defect of *S. boulardii* on galactose. In order to examine the relationship between the truncated Pgm2 and the growth defect on galactose, we introduced an *S. cerevisiae PGM2*-overexpressing plasmid (37) into an auxotrophic *S. boulardii* strain (38). The resulting *S. boulardii* transformant with the *S. cerevisiae PGM2*-overexpressing plasmid showed improved growth on galactose (Fig. 4B and C), indicating that the truncation of Pgm2 in *S. boulardii* is responsible for its inefficient galactose utilization. In order to cross-validate the association between the truncated Pgm2 and inefficient galactose utilization, the point mutation G1278A in *PGM2* was introduced into *S. cerevisiae* strain BY4742 via Cas9-based genome editing. As shown in Fig. 5A, cultures of *S. cerevisiae* BY4742 with the G1278A mutation in *PGM2* (*S. cerevisiae* PGM2-TGA) showed a growth defect on galactose. As the growth phenotype of the *S. cerevisiae* PGM2-TGA strain on galactose was similar to that of the *S. cerevisiae* BY4742 strain with a *PGM2* deletion (*S. cerevisiae* Δ*PGM2*), the truncation of Pgm2 is likely to cause a loss of function. Next, we examined whether the reverse mutation from G1278A to A1278G in *S. boulardii PGM2* could restore growth on galactose. Using Cas9-based genome editing, we created an *S. boulardii* mutant without the early stop codon in *PGM2* (*S. boulardii* PGM2-TGG). As expected, the *S. boulardii* PGM2-TGG strain was able to grow well on galactose. The growth phenotype of the *S. boulardii* strain with deletion of *PGM2* (*S. boulardii* Δ*PGM2*) was similar to that of its parental *S. boulardii* strain with a truncation of Pgm2 (Fig. 5B). Taken together, these results suggest that the point mutation (G1278A) in *S. boulardii PGM2* is responsible for inefficient galactose utiliza-

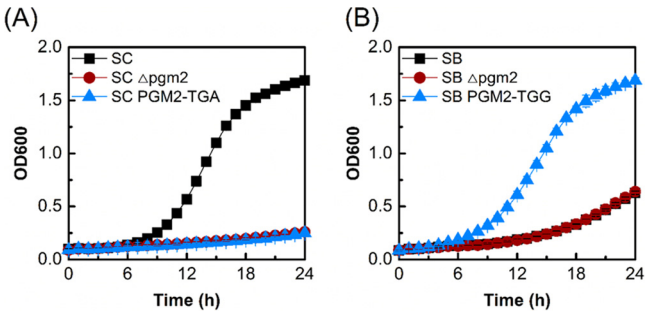

FIG 5 *PGM2* point mutation swapping in *S. cerevisiae* (A) and *S. boulardii* (B). SC △pgm2, *S. cerevisiae strain* BY4742 with the deletion of *PGM2*; SC PGM2-TGA, *S. cerevisiae strain* BY4742 with the point mutation G1278A; SB △pgm2, *S. boulardii* strain with the deletion of *PGM2*; SB PGM2-TGG, *S. boulardii strain* with fixed point mutation, A1278G, in *PGM2*. Results are the means of triplicate experiments; error bars indicate standard deviations and are not visible when smaller than the symbol size.

tion and that the galactose utilization of *S. boulardii* can be enhanced by reversing the G1278A point mutation.

Laboratory evolution of *S. boulardii* after serial subcultures on galactose. Although we learned that the single point mutation G1278A in *PGM2* is responsible for inefficient galactose utilization of *S. boulardii*, we questioned whether the truncated Pgm2 was indeed a rate-limiting step in galactose utilization by *S. boulardii* and whether the detrimental point mutation of Pgm2 could be reversed through laboratory evolution on galactose. We therefore performed serial subcultures of *S. boulardii* on galactose to check whether or not the truncated Pgm2 could be reversed into a full-length Pgm2 via laboratory evolution. As shown in Fig. 6A, the evolved *S. boulardii* colonies grew much faster on galactose than those of the parental strain. A randomly picked colony (SBe1) was tested for its galactose utilization capability using a Bioscreen C system. The evolved *S. boulardii* SBe1 colonies grew well on galactose without exhibiting any galactose toxicity phenotype (Fig. 6B). Next, the PCR product of *PGM2* from the SBe1 strain was sequenced. Interestingly, the *PGM2* gene in the evolved strain

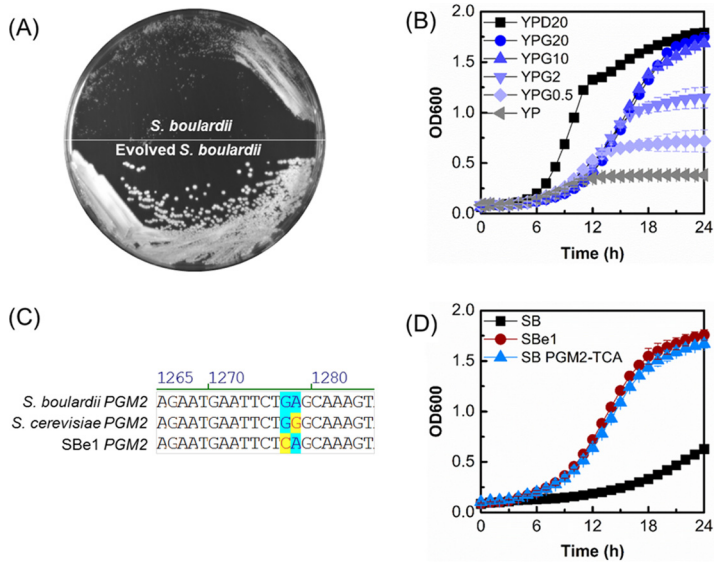

FIG 6 *S. boulardii* that consumed galactose more quickly was obtained after serial subcultures on galactose. (A) The growth of evolved *S. boulardii* on YP plates with galactose as the sole carbon source after 3 days of incubation at 30°C. (B) A randomly picked colony from the plate shown in panel A grew well in galactose medium without exhibiting galactose toxicity. (C) The TGA_{1278} point mutation was switched to $TC_{1277}A$ in evolved *S. boulardii* strain SBe1. (D) The TGA_{1278}-$TC_{1277}A$ switch was responsible for the restored growth of *S. boulardii* on galactose. Results are the means of triplicate experiments; error bars indicate standard deviations and are not visible when smaller than the symbol size.

(SBe1) gained a point mutation (G1277C) (Fig. 6C; see also Fig. S4 in the supplemental material). It is worth mentioning that the stop codon TGA_{1278} in *PGM2* was switched to a codon of serine $TC_{1277}A$ instead of a codon of tryptophan TGG_{1278}, as in *S. cerevisiae PGM2*. This result suggests that a new suppressor mutation (G1277C) can also enhance galactose utilization by *S. boulardii*, as does the A1278G mutation. As the evolved strain (SBe1) might contain other unknown mutations influencing galactose utilization, we reverse-engineered the G1277C mutation in *S. boulardii* through Cas9-based genome editing. The resulting mutant (*S. boulardii* PGM-TCA) showed almost identical phenotypic characteristics to those of the SBe1 strain (Fig. 6D), indicating that the point mutation switching from TGA_{1278} to $TC_{1277}A$ was responsible for the improved galactose utilization capability of the SBe1 strain.

***S. boulardii* strains with a truncated Pgm2 showed growth advantages at 37°C.** As many *S. boulardii* strains have been sequenced, we wondered if the truncation of Pgm2 due to the G1278A mutation also existed in other *S. boulardii* strains isolated from different sources (35, 39–41). As shown in Fig. S5 in the supplemental material, all eight sequenced *S. boulardii* strains (*S. boulardii* strain 17, ATCC MYA-796, ATCC MYA-797, biocodex, EDRL, kirkman, unique28, and Unisankyo) had the same G1278A point mutation in *PGM2*, causing the same truncation of Pgm2. This result suggests that the Pgm2 truncation in *S. boulardii* might confer growth advantages under specific conditions. We therefore examined the growth rates of the parental strain of *S. boulardii* with the truncated Pgm2 and the mutant with the intact Pgm2 (*S. boulardii* PGM2-TGG) under various conditions, using *S. cerevisiae* as a control. The growth rates of these two strains on glucose were similar regardless of growth temperatures. Surprisingly, we found that the *S. boulardii* PGM-TGG strain could not grow well on galactose at 37°C, even though the *S. boulardii* PGM-TGG strain showed improved growth on galactose at 30°C (Fig. 7A). The control strain, *S. cerevisiae* with a functional Pgm2, could grow well on galactose at both 30°C and 37°C, as shown in Fig. S6 in the supplemental material. This indicated that there might be other unknown genes besides *PGM2* that control the growth of *S. boulardii* colonies on galactose under high temperatures. Next, we examined the growth of the *S. boulardii* PGM-TGG strain on glucose at 37°C, using a synthetic complete dextrose (SCD) medium with 20 g/liter of glucose to mimic the natural environment for *S. boulardii* in tropical areas (42). As shown in Fig. 7B, the *S. boulardii* strain with a functional Pgm2 (*S. boulardii* PGM2-TGG) grew more slowly on minimal medium with glucose at 37°C than did the wild-type *S. boulardii* strain with an inactive Pgm2. In order to examine the competition of the *S. boulardii* PGM2-TGG and *S. boulardii* strains under culture conditions mimicking the natural environment of *S. boulardii* in a tropical region, *S. boulardii* PGM2-TGG and its parental *S. boulardii* strain were cocultured in SCD medium at 37°C. As shown in Fig. 7C, the population of the parental *S. boulardii* strain accumulated in SCD medium at high temperatures, more so than that of the *S. boulardii* PGM2-TGG strain. These results indicated that the *S. boulardii* strain with a truncated Pgm2 exhibits growth advantages on glucose at higher temperatures, whereas it cannot grow well on galactose. Considering the natural habitat of *S. boulardii*, the loss of function of Pgm2 caused by the G1278A mutation might be beneficial for the growth of the yeast on the surfaces of fruits in tropical regions.

DISCUSSION

Unusual phenotypic characteristics of *S. boulardii* compared to those of *S. cerevisiae*, especially for inefficient galactose metabolism, have been observed (24–26). However, genetic elements affecting inefficient galactose metabolism—a basic metabolic phenotype—in *S. boulardii* have not been fully understood (39). Even though growth temperature and aeration levels are important factors affecting galactose assimilation by *S. boulardii*, testing conditions for galactose assimilation have not been clearly described in previous reports. Therefore, the previous studies have reported somewhat different findings on galactose utilization by *S. boulardii*. In this study, we identified a

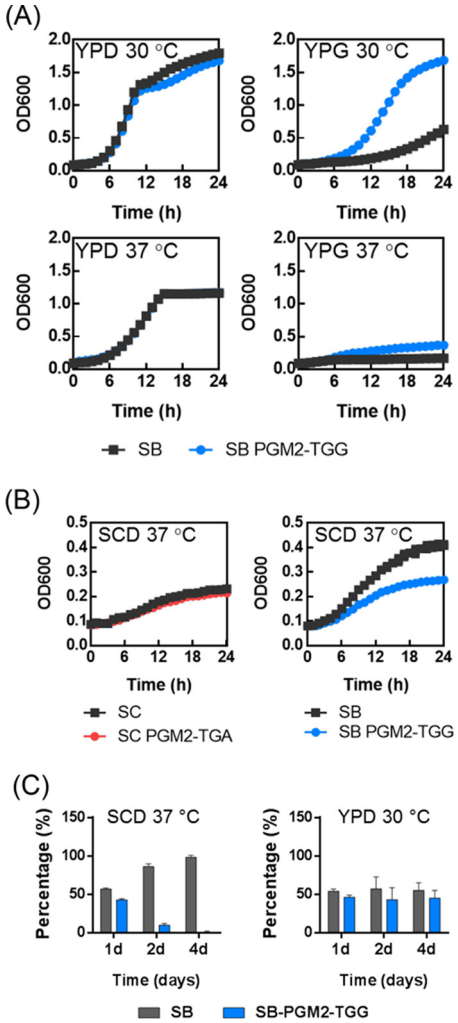

FIG 7 *S. boulardii* with a malfunctional Pgm2 has an evolutional advantage in tropical areas. (A) *S. boulardii* with a fixed Pgm2 (SB PGM2-TGG) could not grow well on galactose under 37°C in complex media. (B) *S. boulardii* with a fixed Pgm2 grew slower than wild-type *S. boulardii* at 37°C in minimal medium. (C) *S. boulardii* could outcompete *S. boulardii* with fixed Pgm2 in a simulated natural environment for *S. boulardii* in tropical areas (SCD medium at 37°C). Results are the means of triplicate experiments; error bars indicate standard deviations and are not visible when smaller than the symbol size.

single-nucleotide mutation in *PGM2*, which codes for a major isoenzyme of phosphoglucomutase, resulting in inefficient galactose metabolism by *S. boulardii*.

In *Saccharomyces cerevisiae*, Pgm2 is known to play a crucial role in controlling the flux through the Leloir pathway, the major catabolic pathway of galactose, probably due to increased conversion of glucose-1-phosphate to glucose-6-phosphate (43). However, we found that a point mutation (i.e., G1278A) in *PGM2* of *S. boulardii* resulted in a truncated form of Pgm2 because the point mutation led to the formation of a stop codon (TGA) (Fig. 4). Occasionally, truncation of enzymes has been used to increase catalytic activities or to increase cytosolic protein expression levels by removing parts that are nonessential for catalytic functions, such as carbohydrate-binding modules or signal peptides (44, 45). However, our findings from the superimposition of predicted protein structures of Pgm2 proteins originating from *S. boulardii* (i.e., truncated Pgm2) and *S. cerevisiae* (i.e., full-length Pgm2) suggested that the truncation of Pgm2 found in *S. boulardii* could affect catabolic activity, because the location of the mutation was close to the active site of Pgm2 (Fig. S3). Specifically, the point mutation led to early termination of protein translation of *PGM2*, resulting in a polypeptide of 426 amino

acids instead of 529 amino acids. According to a previous report, the active site of Pgm2 is inside the "pocket" between the N- and C-terminal regions (46). Therefore, the C-terminal truncation of Pgm2 in *S. boulardii* will lead to an incomplete and defective pocket in the mutated Pgm2, according to the structures predicted by the Phyre2 Web portal (36). Loss of function of the mutated Pgm2 of *S. boulardii* was further confirmed by comparing the galactose fermentation rates of native *S. boulardii* and *PGM2*-deleted *S. boulardii* (Fig. 5C). The fermentation profiles by two strains under galactose conditions were identical. When Pgm2 was inactivated, both strains were able to grow on galactose slowly, as *S. boulardii* has an isoform Pgm1.

Next, we examined the essentiality of Pgm2 activity for efficient galactose utilization by *S. boulardii* through making a reverse mutation in the mutated *PGM2* and by adaptive evolution under galactose of *S. boulardii* harboring the point mutation G1278A in *PGM2* conditions (Fig. 5 and 6). In general, the major sources of galactose in nature are galactan polysaccharides, such as carrageenan, porphyran, and agar, which are found in marine environments (47), and lactose, found in milk from mammary glands. Therefore, inefficient galactose fermentation by *S. boulardii* is presumed to result from long-term adaptation of *S. boulardii* to its ecological niche (e.g., surfaces of mangosteen and lychee fruits in a tropical area), where galactose is rarely present. As genomic sequences of more *S. boulardii* strains (*S. boulardii* strain 17, ATCC MYA-797, biocodex, EDRL, kirkman, unique28, and Unisankyo) are available (35, 39–41), we compared the sequences of *PGM2* from different sources of *S. boulardii* strains. Interestingly, all of the sequenced *S. boulardii* strains have the same point mutation in *PGM2*, G1278A, indicating that the *PGM2* sequence is evolutionarily conserved in *S. boulardii*.

The above-mentioned findings then led us to raise a question regarding the effect of the *PGM2* mutation in *S. boulardii* on its metabolism of other prevalent sugars. We performed growth competition experiments between the parental *S. boulardii* strain with a truncated Pgm2 and a mutant *S. boulardii* strain (*S. boulardii* PGM-TGG) with a full-length Pgm2, using glucose or galactose as a sole carbon source, under 30°C and 37°C temperatures (Fig. 7). When similar numbers of the *S. boulardii* and *S. boulardii* PGM-TGG cells were coinoculated, the *S. boulardii* PGM-TGG strain became a dominant population on galactose at 30°C, but the *S. boulardii* strain became a dominant population on glucose at 37°C. These results suggest that the mutated Pgm2 causes inefficient galactose utilization. However, it can be beneficial for *S. boulardii* cells to grow on glucose, especially at higher temperatures. Although we confirmed that the inferior galactose utilization by *S. boulardii* was caused by the truncated Pgm2, potential mechanisms by which the truncated Pgm2 provides growth advantages under high temperatures remained unclear.

Our results showed that efficient galactose metabolism is associated with the G1278A mutation in *PGM2* in *S. boulardii*. Also, we found that the G1278A mutation in *PGM2* is also associated with growth phenotypes of *S. boulardii* in minimal media at high temperature. Therefore, we speculate that the mutation in *S. boulardii* might allow a longer transition time in the human gut, where the temperature is 37°C. The longer transition time and enhanced growth at 37°C of *S. boulardii* cells have been proposed as putative determinants of probiotic activities by *S. boulardii* (22). Future animal experiments will be necessary to find the relationship between the *PGM2* mutation and the transition time of *S. boulardii* in the human gut.

In conclusion, we report why *S. boulardii* exhibits inefficient galactose metabolism in this study. We confirmed that *S. boulardii* is capable of consuming galactose at a relatively lower rate than that of a representative *S. cerevisiae* strain, BY4742. We found that the low galactose utilization of *S. boulardii* was caused by a point mutation (G1278A) in *PGM2*. This point mutation led to early termination of the protein translation of Pgm2, resulting in a truncated Pgm2, which is responsible for low galactose metabolism. Lastly, our results provide information regarding the biological functions of Pgm2 and additional effects of truncation of Pgm2 on utilization of other sugars, such as glucose, in the yeast *S. boulardii*.

TABLE 1 Primers, plasmids, and strains used in this study

Primer, plasmid, or strain	Sequence or description[a]	Source (reference)
Primers		
gRNA-u	AATGAATTCTGGGCAAAGTAgttttragagctagaaatagcaag	This study
gRNA-d	TACTTTGCCCAGAATTCATTgatcatttatctttcactgcgga	This study
Donor1-u	catccggagaacgaagcttctattaagacgatacagaatgaattctgAgcaaagtacgg	This study
Donor1-d	ttcaacttttttcaaaatcataacgagtgaagaaagtacggccgtactttgcTcagaatt	This study
Donor2-u	catccggagaacgaagcttctattaagacgatacagaatgaattctgGgcaaagtacgg	This study
Donor2-d	ttcaacttttttcaaaatcataacgagtgaagaaagtacggccgtactttgcCcagaatt	This study
Donor3-u	catccggagaacgaagcttctattaagacgatacagaatgaattctCagcaaagtacgg	This study
Donor3-d	ttcaacttttttcaaaatcataacgagtgaagaaagtacggccgtactttgctGagaatt	This study
PGM2-u	catggaagctattccagag	This study
PGM2-d	accgttggttcttcagttcc	This study
PGM2-Seq-u	ccagcatggtcttctgtctac	This study
PGM2-Seq-d	gtacctgacaatcttagaac	This study
Plasmids		
Cas9-NAT	p414-TEF1p-Cas9-CYC1t-NAT1	Addgene 64329 (50)
p42K-gPGM2	pRS42K carrying guide RNA for *PGM2* deletion	This study
Strains		
S. boulardii	*S. boulardii* (ATCC-MYA-796)	ATCC (38)
S. cerevisiae	*S. cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*)	EUROSCARF (48)
S. cerevisiae Δpgm2	*S. cerevisiae* BY4742 with *PGM2* deletion	EUROSCARF (48)
S. boulardii Δpgm2	*S. boulardii* (ATCC-MYA-796) with *PGM2* deletion	This study
S. boulardii SBe1	Isolated from serial subcultures of *S. boulardii* on galactose	This study
S. cerevisiae PGM2-TGA	*S. cerevisiae* with mutated *PGM2* (G1278A); stop codon	This study
S. boulardii PGM2-TGG	*S. boulardii* with mutated *PGM2* (A1278G); mimic *S. cerevisiae*	This study
S. boulardii PGM2-TCA	*S. boulardii* with mutated *PGM2* (G1277C); mimic SBe1	This study

[a]The capital letters are 20 base pairs of targeting sequences of guide RNA.

MATERIALS AND METHODS

Strains and media. *Escherichia coli* TOP10 cells were used for the construction and propagation of plasmids. *E. coli* cells were grown in Luria-Bertani medium (5 g/liter yeast extract, 10 g/liter tryptone, and 10 g/liter NaCl; pH 7.0) at 37°C, and ampicillin (100 μg/ml) was added for selection when required. The *S. boulardii* strain used in this study was ATCC MYA-79 (ATCC, Inc.) (38). *S. cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) was used as a control strain. Strain BY4742 with a *PGM2* deletion (*S. cerevisiae* Δpgm2) was from the Yeast Knockout (YKO) Collection (EUROSCARF) (48). Yeast strains were grown on yeast extract-peptone-dextrose (YPD) medium (10 g/liter yeast extract and 20 g/liter peptone containing 20 g/liter glucose) at 30°C or 37°C, as indicated. Yeast strains transformed with plasmids containing antibiotic markers were propagated on YPD plates supplemented with the corresponding antibiotics.

Plasmid and strain construction. The plasmid containing guide RNA targeting *PGM2* (p42K-gPGM2) (Table 1) was constructed by reverse PCR of p42K-gCS8 (38), using the primers gRNA-u and gRNA-d (Table 1). The primers Donor1-u/d, Donor2-u/d, and Donor3-u/d, used for donor DNA to swap the *PGM2* point mutation, are listed in Table 1. The point mutation swapping was conducted through CRISPR-Cas9 based technology (49), using the plasmid Cas9-NAT (38, 50) (Table 1). The resulting transformants of *PGM2* point mutation swapping were first tested for growth on galactose. The PCR products of *PGM2* were amplified from the colonies, which could grow on galactose, using the primers PGM2-u and PGM2-d (Table 1). The PCR products of swapped *PGM2* were further confirmed by sequencing (ACGT, Inc., Wheeling, IL), using the primers PGM2-Seq-u and PGM2-Seq-d (Table 1).

Yeast growth measured via Bioscreen C. The automated Bioscreen C plate reader (Growth Curves USA, Piscataway, NJ) was used to evaluate the growth of yeast strains. The yeast cells were precultured in YPD for 24 h, diluted to at initial optical density at 600 nm (OD_{600}) of 0.1, and incubated in a Honeycomb 2 multiwell plate at 30°C or 37°C, as indicated. An OD_{600} reading was measured every hour over a period of 24 h or longer. The medium for the Bioscreen C test was YPG (yeast-peptone [YP] medium containing galactose as the carbon source), with different galactose concentrations as indicated (i.e., YPG20 represents YP medium with 20 g/liter of galactose), or synthetic complete (SC) medium (6.7 g/liter of yeast nitrogen base with ammonia sulfate and amino acids) with 20 g/liter of glucose (SCD).

Galactose fermentation and metabolite analysis. Galactose fermentation was performed by inoculating the overnight preculture (5 ml of YPD medium) into 20 ml of YPG with an initial OD_{600} of 1.0 in a 125-ml Erlenmeyer flask and incubating at 30°C or 37°C and 100 or 250 rpm as indicated. OD_{600} was measured by spectrophotometer (Biomate 5; Thermo, New York, NY), and extracellular metabolite concentrations were measured by high-performance liquid chromatography (HPLC; Agilent Technologies 1200 Series). The HPLC system was equipped with a Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., Torrance, CA) and a refractive index detector (RID). The column was eluted with 0.005 N H_2SO_4 at a flow rate of 0.6 ml/min at 50°C.

Serial subcultures of *S. boulardii* on galactose. *S. boulardii* was subjected to serial subcultures on galactose (YP medium with 20 g/liter of galactose) at an initial OD_{600} of 0.1. A fully grown culture was repeatedly transferred to a new culture after galactose was consumed. After 10 serial subcultures, individual colonies were isolated from YPG agar plates (YP medium with galactose as a sole carbon source). After 3 days of incubation at 30°C, the growth rates of the evolved *S. boulardii* and its parental strain were compared.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02858-17.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

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