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Sugar Utilization-Associated Food-Grade Selection Markers in Lactic Acid Bacteria and Yeast

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Abstract

This comprehensive review explores the development of food-grade selection markers in lactic acid bacteria and yeast; some of their strains are precisely defined as safe microorganisms and are crucial in the food industry. Lactic acid bacteria, known for their ability to ferment carbohydrates into lactic acid, provide essential nutrients and contribute to immune responses. With its strong fermentation capabilities and rich nutritional profile, yeast finds use in various food products. Genetic engineering in these microorganisms has grown rapidly, enabling the expression of enzymes and secondary products for food production. However, the focus is on ensuring safety, necessitating food-grade selection markers. Traditional antibiotic and heavy metal resistance selection markers pose environmental and health risks, prompting the search for safer alternatives. Complementary selection markers, such as sugar utilization markers, offer a promising solution. These markers use carbohydrates as carbon sources for growth and are associated with the natural metabolism of lactic acid bacteria and yeast. This review discusses the use of specific sugars, such as lactose, melibiose, sucrose, D-xylose, glucosamine, and N-acetylglucosamine, as selection markers, highlighting their advantages and limitations. In summary, this review underscores the importance of food-grade selection markers in genetic engineering and offers insights into their applications, benefits, and challenges, providing valuable information for researchers in the field of food microbiology and biotechnology.

K e y w o r d s: food-grade, selection marker, GRAS, genetic engineering, lactic acid acid bacteria, yeast

Introduction

Partial strains of lactic acid bacteria and yeasts are defined as probiotics, microorganisms with health-promoting effects on humans and animals. These bacteria and fungi are Generally Recognized as Safe (GRAS) microorganisms among prokaryotes (Duche et al. 2023) and eukaryotes (Liu and Cheng 2023), respectively, and they have been defined by the Food and Agriculture Organization/World Health Organization as probiotics (Iqbal et al. 2014). Therefore, they are widely used in the food industry. Lactic acid bacteria is a collective term representing gram-positive bacteria that ferment carbohydrates to produce lactic acid (Dong and Li 2012). They can provide proteases, amino acids, vitamins, and other growth substances to the animal body, promoting the individual's growth and development (Scholz-Ahrens et al. 2007). Additionally, lactic acid bacteria can activate the immune mechanisms of bodily fluids and cells in individuals, contributing to immune responses such as apoptosis in tumor cells (Yuan et al. 2011). Yeast is a fungus with a strong fermentation ability, rich nutritional value, and stable characteristics, including its gene expression regulation mechanism and protein modification systems. It can hydrolyze polysaccharides or proteins into monosaccharides or amino acids, facilitating animal digestion and nutrient absorption (Marklinder et al. 1995). Due to the various nutrients present in yeast, it is widely used in the processing and production of condiments, flour products, spices, and other foods (Zhang et al. 2023).

Currently, the genetic engineering of lactic acid bacteria and yeast is developing rapidly. Various enzymes, vitamins, and secondary products related to food production can be expressed and transformed in large quantities through foreign gene fragment insertion. However, establishing these genetically engineered microorganisms must be based on a premise that ensures human health and environmental protection. Therefore, the required reaction substrates, inducers,

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and screeners need to be food-grade. Selection markers are essential components for constructing genetically engineered microorganisms. Selection markers for resistance to antibiotics or heavy metals have been used in most previous genetic engineering programs. However, neither of these two markers are food-grade selection markers. Antibiotic resistance selection markers easily spread with the changing seasons and endanger human health and the whole biosphere (Trombert 2015). Heavy metal resistance selection markers can cause the host cells to introduce toxic heavy metal ions in the culture process, which can affect the health of individuals via heavy metal toxicity in the gastrointestinal tract (Plavec and Berlec 2020).

Establishing safe and stable selection markers in food-grade expression vectors is a hot topic in current research (Huang and Li 2007; He et al. 2012). Presently, only dominant and complementary selection markers can be used. Dominant and antibiotic resistance selection markers share the same principle, and bacteriocins are representative of these screening agents (Xiang et al. 2007), but there are not many types of these selection markers. Complementary selection markers affect the host microorganism through the natural environment or artificial means and change their traits, lowering their survival in the corresponding basic or specific medium. Then, if the strain can grow normally by converting some specific selection marker genes related to the metabolic pathway, the transformants are obtained. D-alanine, threonine, purines, pyrimidines, and other substances that are harmless to humans have been used as screeners to establish food-grade expression vectors (He et al. 2012). Sugar utilization markers are complementary selection markers, and their screeners include lactose, melibiose, sucrose, D-xylose, glucosamine, and N-acetylglucosamine. Typically, lactic acid bacteria and yeast use carbohydrates as the carbon source for their growth during cultivation. Using sugar metabolism-associated key genes as selection markers and the corresponding carbon sources as screeners has the following two advantages (Tian et al. 2017): first, the transformant can obtain sufficient energy on the screening plate, rather than being in a hungry state, and second, in the case of non-purification, carbohydrates are edible, whereas in the case of purification, the purified protein exhibits no toxicity or sensitization. Therefore, when the inducer is of food-grade, genetically engineered microorganisms with safe and stable food-grade microorganisms as hosts, expression vectors containing sugar utilization marker elements, and no non-food-grade functional DNA can not only meet the primary conditions of a GRAS microbial expression system (Huang and Li 2007), but also ensure the safety of the produced food.

This review aims to summarize the experimental results of food-grade expression vector construction with various sugar utilization markers as screening elements using lactic acid bacteria and yeast as host microorganisms and compare and analyze them.

Lactose

Among all the sugar utilization markers, lactose has been widely used as the screener for constructing food-grade expression vectors (Table I). Because of the presence of the lactose metabolism-associated coding gene on the expression vector, after successful transformation, the recombinant microorganisms grew yellow or blue colonies on the screening plate containing acid-base indicators (Platteeuw et al. 1996), such as bromocresol purple or 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

All the lactic acid bacteria can metabolize lactose (Fan et al. 2006). Therefore, the β-galactosidase coding gene of lactic acid bacteria must be knocked out via gene editing techniques so the recipient bacterium can mutate into Lac-phenotype. Lac+ phenotype can be recovered when the recombinant plasmid containing the β-galactosidase coding gene is imported to

Table I
Lactose utilization markers.

Recombinant vector	Marker gene	Host	Strain number	Reference
*pMYL1-βga*l	*βgal*	*Lactobacillus acidophilu*s	ADH	Li et al. 1996
pBG10	*βgal*	*Lactobacillus helvetic*us	SBT2195	Hashiba et al. 1992
PNZ8149-Human FGF21	*lacF*	*Lactococcus lact*is	NZ3900	Cao et al. 2020
pNZ2116, *pNZ2118*, *pNZ2119*	*lacF*	*Lactococcus lacti*s	NZ3900	Platteeuw et al. 1996
pTN	*lacF*	*Lactococcus lacti*s	NZ3000	Tagliavia et al. 2019
PMG36e-lacZ	*lacZ*	*Lactobacillus acidophilu*s	ATCC® 4356™	Deng et al. 2011
pMG36m	*lacL*, *lacM*	*Lactiplantibacillus plantarum*	LP01	Wang et al. 2023
pIlacgus, pIlacilv	*lacF*, *lacG*	*Lacticaseibacillus casei*	CECT 5276	Gosalbes et al. 2000

such defective strain. β-Galactosidase is a key excision enzyme that catalyzes lactose hydrolysis to generate glucose and galactose. Some β-galactosidase also exhibit transglycosylation activity and can transfer galactosyl groups to various substrates to generate transglycosylation products with various prebiotic functions (Posno et al. 1991b; Delgado-Fernandez et al. 2020). β*gal* gene from *Lactobacillus delbrueckii* subsp. *bulgaricus* (Hashiba et al. 1992; Lin et al. 1996), *lacF* gene from *Lactococcus lactis* (Platteeuw et al. 1996; Cao et al. 2019; Tagliavia and Nicosia 2019), *lacZ* gene from *Lactobacillus acidophilus* (Deng et al. 2011), and *lacLM* gene from *Lactiplantibacillus plantarum* (Wang et al. 2023) are all genes encoding β-galactosidase. Studies have shown that these genes can be used as selection markers for lactose metabolism. Some lactic acid bacteria, such as *Lacticaseibacillus casei*, have two lactose metabolic pathways (Vera et al. 2019), one of which is the β-galactosidase metabolism (*lacF*) pathway. The other pathway involves the phosphorylation of ingested exogenous lactose to 6-phosphate lactose, followed by the hydrolysis of phospho-β-galactosidase (*lacG*) into 6-phosphate galactose and glucose. Therefore, it is feasible to knockout *lacF* and *lacG* genes from the genome and use them as selection markers in expression vectors (Gosalbes et al. 2000).

Using inexpensive lactose as a selection marker can save the process cost and facilitate the co-production of milk-related products because lactose is also present in milk. After successful construction, the recombinant microorganisms and their products can be combined with some commonly used milk-based media to produce various healthy, nutritious, or better-tasting foods. Using the lactose promoter in plasmid vectors with lactose-selective markers and inducing with lactose can help achieve good expression (Wang et al. 2023); this construction can exhibit dual effects of the screening of exogenous lactose and lactose-selective marker expression. Selecting the target host bacteria would be a better and more straightforward method than using lactic acid bacteria with only β-galactosidase (lacF) activity, knocking out its lacF gene and constructing it into a plasmid vector (MacCormick et al. 1995; Platteeuw et al. 1996). There levant research analysis results show that the selection marker of β*gal* is more applicable to the integration with chromosomes (Hashiba et al. 1992; Lin et al. 1996) rather than exist in the host cell with modality of recombinant plasmid; the *lacLM* coding protein is the heterodimer (Wang et al. 2023) formed by large subunit and small subunit coded by *lacL* and *lacM* gene. It is undoubtedly difficult to construct such recombinant carrier on account of relatively long coding gene; the plasmid vectors labeled with the *lacG* gene exhibits incompatibility with wild-type lactic acid bacterial strains (Takala et al. 2003). The selection marker of *lacZ* is not often reported and may be researched more deeply. Currently, the selection marker of many commercialized food-grade vectors competent for metabolized lactose is from the *lacF* gene.

Melibiose

Similar to β-galactosidase, α-galactosidases are also a type of excision enzyme that can be divided into two categories: one that targets galactomannan and hydrolyzes low-Mr substrates to varying degrees and the other that resolves melibiose into galactose and glucose (Shibuya et al. 1998). Present studies have shown that the α-galactosidase selection marker exhibits the latter's activity, which has multiple coding genes from different species. There are mainly four types of markers that are used for genetic engineering construction (Table II): *aga* gene from *Lactococcus raffinolactis* (Boucher et al. 2002; Labrie et al. 2005; Xu et al. 2007; Gu et al. 2014); *mel1* gene from *Saccharomyces cerevisiae* (Guo et al. 2010; Pontes et al. 2020); *meiA* gene from *L. plantarum* (Jeong et al. 2006); and *aglL* gene from *Bifidobacterium longum* (Sridhar et al. 2006). In contrast to lactose-selection markers, most lactic acid bacteria and yeast do not exhibit α-galactosidase activity; therefore, if melibiose is used as the only carbon source for constructing

Table II
Melibiose utilization markers.

Recombinant vector	Marker gene	Host	Strain number	Reference
pRAF800, *pRAF803*	*aga*	*Lactococcus lactis*	MG1363, SMQ-741, SMQ-561, IL1404	Boucher et al. 2002
pMG36-aga-amy	*aga*	*Lactococcus lactis*	ML23	Gu et al. 2014
pRAF301	*aga*	*Streptococcus thermophilus*	RD733	Labrie et al. 2005
pRNA48-OprF/H, *pRNV48-OprF/H*	*aga*	*Lactococcus lactis*	NZ3900	Xu et al. 2007
YGMPNA-PM	*mel1*	*Saccharomyces cerevisiae*	MS-1	Guo et al. 2010
pFMN30	*meiA*	*Lactococcus lactis*	NZ9800	Jeong et al. 2006
pSUW711	*aglL*	*Lactococcus lactis*	JLS400	Sridhar et al. 2006

the food-grade selection markers, then there is no need to modify the host genome.

The production of α-galactosidase has a particular physiological importance, as many soy products contain melibiose, which humans and monogastric animals cannot digest and metabolize, and its excessive consumption may lead to stomach pain, nausea, and diarrhea (Jeong et al. 2006). Because both lactic acid bacteria and yeast are a part of the native intestinal microbiota, we can establish "α-galactosidase-producing, food-grade, genetically engineered microorganisms as health-care products to improve the digestion of soy products. "α-Galactosidase is also used as a starter culture for soybean milk fermentation (Sridhar et al. 2006); therefore, establishing such genetically engineered microorganisms is beneficial for producing better soybean products by the metabolizing undesirable byproducts during the production process.

Temperature dramatically affects "α-galactosidase activity in different species. A study showed that the recombinant plasmid *pRAF301* in *Streptococcus thermophilus* harboring the *aga* gene lost its melibiose-metabolizing activity at 42°C, which is an optimal temperature for the recipient *S. thermophilus*, but not at 37°C (Labrie et al. 2005). This situation was closely related to the optimal culture temperature (37°C) for the original host of the *aga* gene, namely *L. raffinolactis*. A similar observation was made for the *meiA* gene of *L. plantarum*, but at the optimal culture temperature of 30°C, where the α-galactosidase activity was considerably higher than that at 37°C (Silvestroni et al. 2002).

Using the "α-galactosidase-encoding gene as a food-grade selection marker to construct genetically engineered strains may make these methods more convenient. However, it is important to note that in host microorganisms using melibiose as the carbon source, the intracellular acquisition of melibiose requires permease (*galA*), which transports melibiose smoothly to the intracellular region (Huang and Li 2007); the absence of *galA* gene can impede the activity of melibiose metabolism markers, as the intracellular melibiose content would be lacking. Therefore, the selection of recipient bacterium is restricted. Early studies have suggested that the end of the *aga* gene needs to be concatenated with its transcriptional regulatory gene (*galR*) for the recombinant strain to utilize melibiose for acid production (Boucher et al. 2002), but in the later studies, some recipient strains cloned the *aga* gene and were able to grow without *galR* gene regulation (Labrie et al. 2005; Xu et al. 2007; Gu et al. 2014). Recombinant yeast with the *meL1* gene cannot be used in the alt beer industry (Guo et al. 2010). The *meiA* gene in the recombinant bacteria exhibits a high activity when combined with its promoter (Jeong et al. 2006).

In contrast, the *aglL* gene exhibits a low activity and must be provided additional promoters (Sridhar et al. 2006). Additionally, the price of high-purity melibiose is considerably high, and if it is used for industrial production, it may not be profitable.

Sucrose

The resident plasmid of PPE1.0, a subspecies of *Pediococcus pentosaceus*, contains two metabolic genes related to sucrose metabolism, namely *scrA* and *scrB* (Guo et al. 2020). The *scrA* gene encodes a phosphotransferase system (PTS)-dependent sucrose transport system protein that transports extracellular sucrose into the cell and converts it into sucralose-6-phosphate (Wang et al. 2001). The *scrB* gene encodes sucralose-6-phosphate hydrolase, which hydrolyses sucralose-6-phosphate to generate glucose-6-phosphate and fructose (Gonzalez and Kunka 1986), helping the cell's expected growth.

Cloning the scrA and scrB genes on the plasmids can establish a food-grade selection marker with sucrose as the only carbon source (Table III). In a study, the two coding genes, namely *scCrA* and *scrB* genes, and the Campbell-type ori+ vector were used to construct an integrated vector and integrated into the genome of *L. delbrueckii* subsp. *lactis* and had limited sucrose-metabolizing ability; the feasibility of the tag was assessed in the M17 agar plates with the sucrose (Leenhouts et al. 1998a). In a subsequent study, proline iminopeptidase, commonly used in cheese, was successfully expressed in this type of recombinan *L. delbrueckii* subsp. *lactis* by adding the P23 promoter (Leenhouts et al. 1998b).

Although sucrose is a considerably inexpensive sugar in the fermentation industry, studies have shown that this selection system requires high copy numbers of sucrose metabolism-associated genes to obtain sufficient expression for the stable growth of the host bacteria (Huang and Li 2007). Moreover, the recom-

Table III
Sucrose utilization markers.

Recombinant vector	Marker gene	Host	Strain number	Reference
pINT124, *pINT125*, *pINT129, pINT142*	*scrA*, *scrB*	*Lactococcus lactis*	LL108, LL302	Leenhouts et al. 1998a
pUG239	*scrA*, *scrB*	*Lactococcus lactis*	MG1363	Leenhouts et al. 1998b

binant microorganisms grow very slowly in a sucrose-containing culture medium, which may not be suitable for large-scale fermentations (Leenhouts et al. 1998b). However, further studies are needed to improve and optimize this selection system.

D-Xylose

In many bacteria utilizing D-xylose as a carbon source, which have four genes involved in D-xylose catabolism, namely *xylR* (positive regulator), *xylA* (D-xylose isomerase-coding gene), *xylB* (D-xylulose kinase-coding gene) and *xylT* (D-xylosyltransferase-coding gene) (Lokman et al. 1991). The intermediates D-xylulose and D-xylulose-5-phosphate play an important role in the 6-phosphogluconate and the pentose phosphate pathways (Lokman et al. 1991). Concerning genetic engineering, the D-xylose metabolism-associated genes commonly utilized in genetic transformation were obtained from the *Clostridioides* (Chen et al. 2015) (for screening the recombinant plants) and the *Lactiplantibacillus pentosus* (Posno et al. 1991b) (for screening the recombinant microorganisms).

Owing to the lack of one or multiple D-xylose metabolism-associated genes, most lactic acid bacteria species (Lokman et al. 1991) and yeast cannot utilize D-xylose for fermentation (Jetti et al. 2019). This feature can be exploited to construct food-grade selection marker vectors using D-xylose as the sole carbon source through genome sequence analysis of relevant coding genes lacking in the targeted host (Table IV). The *xylA*, *xlyB*, and *xylR* genes were subsequently added to the Escherichia coli-Lactobacillus shuttle plasmid *pLP3537* through transformation, and the *L. casei* and *L. plantarum* were able to grow in the presence of D-xylose as a carbon source (Posno et al. 1991a). Using the Easy-Clone 2.0 system, the *xylA* and *xylB* genes and the five key genes of the pentose phosphate pathway were integrated into the host genome of *S. cerevisiae*, enabling its better application in large-scale industrial fermentation (Stovicek et al. 2015).

D-xylose is widely used in food processing and starch industries and is entirely safe (Tian et al. 2017). However, its limitations include the large gene fragments that may hinder the normal replication of plasmids in some host cells. Moreover, similar to melibiose, D-xylose, which is highly pure, is considerably expensive (Huang and Li 2007). For the use of D-xylose as a sugar utilization marker to construct a genetically engineered strain, the plasmid load and the value of the fermented product need to be evaluated.

Glucosamine and N-Acetylglucosamine

D-fructose-6-phosphate aminotransferase is the key catalytic enzyme in the hexosamine metabolic pathway, and its encoding genes (the *glmS* gene in prokaryotes and the *gfat* gene in eukaryotes) are widely present in microorganisms (Görke and Vogel 2008). It is also an important metabolic control point in the biosynthesis of large glucosamine-containing molecules (Milewski 2002; Durand et al. 2008). It utilizes glutamine as a nitrogen donor to catalyze the formation of D-glucosamine 6-phosphate (GlcN6P) from fructose-6-phosphate (Marshall et al. 1991). The absence of fructose-6-phosphate aminotransferase is lethal for microorganisms (Chen et al. 2012); this protein is related to cell wall synthesis, making it an essential protein (Shang et al. 2014).

After deleting the glmS/gfat gene, the deficient microorganism needs exogenous glucosamine or N-acetylglucosamine in the culture medium to grow. When both are introduced into the cells in the absence of fructose-6-phosphate aminotransferase, the former is converted to GlcN6P, and the latter is converted to N-acetylglucosamine-6-phosphate (GlcNAc6P), which can also be obtained using GlcN6P as a substrate. Otherwise, such defective strains cannot grow properly (Bulik et al. 2003; Wu et al. 2011; Chen et al. 2012). In contrast to the sugars mentioned above as utilization markers, when the deficient strains were introduced with the expression vector containing the *glmS*/*gfat* gene, the recombinant strains can normally grow in the basal medium similar to the wild-type strains. Thus, the recombinant bacteria possessing this selection marker do not need a single carbon source as the selective pressure to maintain the vector stability.

Notably, yeast extract and tryptone should be removed from the culture plate because both contain trace amounts of glucosamine, which can affect the

Table IV
D-xylose utilization markers.

Recombinant vector	Marker gene	Host	Strain number	Reference
pLP3537-xyl	*xylA*, *xylB*, *xyl*R	*Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*	ATCC® 393™, NCDO 1193	Posno et al. 1991
δTy2:TEF1p-CpXylA/ TDH3p-PsXYL3	*xylA*, *xylB*	*Saccharomyces cerevisiae*	Ethanol Red, CLIB382, CEN.PK113-7D	Stovicek et al. 2015

Table V
Glucosamine and N-acetylglucosamine utilization markers.

Recombinant vector	Marker gene	Host	Strain number	Reference
pSIPH497, *pSIPH498*	glmS	*Lactiplantibacillus plantarum*	NZ5332	Chen et al. 2018
pREP-AGCX	gfat	*Schizosaccharomyces pombe*	YHL6381	Wu et al. 2011

post-transformation screening of defective or recombinant strains (Chen et al. 2012). Some lactic acid bacteria, such as *L. plantarum*, are reported to have two *glmS* genes (*glmS1* and *glmS2*) in their genome; however, the amino acid sequence encoded by *glmS2* gene has only 13–20% homology with that encoded by the identified *glmS* gene, and its functions are currently unclear (Chen et al. 2012). Therefore, simply knockout *glmS1* gene can affect the normal growth of the strain in the basic culture medium. Presently, only a few studies have used glucosamine or N-acetylglucosamine as a food-grade screening marker to establish genetically engineered bacteria (Table V); however, further studies are needed to verify whether this screening method can affect the normal growth of different bacterial species. The advantage of glucosamine or N-acetylglucosamine as food-grade selection markers is that the screening plate after strain recombination does not require indicators, and only basic nutrients need to be provided to the recombinant microorganisms; therefore, it has a good research value.

Conclusion

With the advancements in biotechnology, the biological safety regarding genetic transformation has garnered much attention. As edible microorganisms, lactic acid bacteria and yeast have bright application prospects. Presently, many food-grade, genetically engineered microorganisms have been successfully constructed, and they have been applied in health care, fermentation, food processing, and other fields (Abrha et al. 2023; González-Orozco et al. 2023; Van de Voorde et al. 2023). The selection markers on vectors are an important part of the selection system for recombinant microorganisms. However, suppose the engineered microorganisms are cultured with a single carbohydrate as the carbon source for energy. In that case, plant growth may not be better than that when cultured with glucose, which is the most basic carbon source. Lactose and sucrose require a vector with a selection marker with a high copy number to obtain a good screening effect, and melibiose and xylose are expensive. They can increase production costs (Xiang et al. 2007).

The glucosamine-6-phosphate synthase-encoding gene is essential for the growth of various eukaryotic and prokaryotic microorganisms and is a newly established food-grade selective marker. It can compensate for the shortcomings of other sugar utilization markers, and engineered microorganisms can use multiple carbon sources to provide nutrition in large-scale industrial fermentation production without selection pressure. Therefore, the selection system with the glucosamine-6-phosphate synthase gene has great research value, and future studies may focus on exploiting its potential in various industries.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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