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Bacterial α -Diglucoside Metabolism: Perspectives and Potential for Biotechnology and Biomedicine

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Abstract

In a competitive microbial environment nutrient acquisition is a major contributor to the survival of any individual bacterial species, and the ability to access uncommon energy sources can provide a fitness advantage. One set of soluble carbohydrates that have attracted increased attention for use in biotechnology and biomedicine are the α -diglucosides. Maltose is the most well studied member of this class, however the remaining four less common α -diglucosides (trehalose, kojibiose, nigerose, and isomaltose) are increasingly used in processed food and fermented beverages. The consumption of trehalose has recently been shown to be a contributing factor in gut microbiome disease as certain pathogens are using α -diglucosides to outcompete native gut flora. Kojibiose and nigerose have also been examined potential prebiotics and alternative sweeteners for a variety of foods. Compared to the study of maltose metabolism our understanding of the synthesis and degradation of uncommon α -diglucosides is lacking and several fundamental questions that remain unanswered, particularly in regards to the regulation of bacterial metabolism for α -diglucosides. Therefore this minireview attempts to provide a focused analysis of uncommon α -diglucoside metabolism in bacteria, and suggests some future directions for this research area that could potentially accelerate biotechnology and biomedicine developments.

Keywords

α -diglucoside; carbohydrate active enzyme; isomaltose; kojibiose; maltose; nigerose; sakibiose; trehalose

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INTRODUCTION

One major determinant of bacterial survival is the ability to acquire and utilize nutrients obtained from their environment. Competition to obtain nutrients is a major driver of bacterial community development and affects both species composition and metabolic potential (Guignard et al. 2017). Bacterial species that exploit diverse nutrient sources gain a competitive advantage over those that are selective or restrictive in nutrient acquisition. For example, within the human gut microbiome alterations in diet greatly altered the composition of gut microbiota (Sanz et al. 2005; Holscher 2017). Of particular biomedical interest, it was recently shown that the ability of some pathogenic bacteria to use rare nutrient sources is a determinant of disease establishment and progression (Sanz et al. 2005; Chen et al. 2007). At the same time, a deeper understanding of how bacteria compete for nutrients has resulted in two major therapeutic approaches, probiotics that attempt to introduce new bacteria to an environment, and prebiotics that aim to alter the composition of bacteria that are already existing in an environment (Holscher 2017; Tsai et al. 2019). A deeper understanding of the nutrient utilization constraints of bacteria is not only important for enhancing human health, but for also to advance biotechnology and industrial applications. Engineering bacterial strains to utilize rare nutrient sources has been shown to increase feedstock conversion efficiency and increased metabolite yields (Keasling 2010; Cairns et al. 2019). As a consequence, characterizing the mechanisms that bacterial species employ to metabolize uncommon nutrient sources provides insight into the physiological functions that are important from both ecological and industrial perspectives (Peralta-Yahya et al. 2012).

One metabolite that is drawing increased interest due to its biomedical and industrial importance is the α -diglucoside. These disaccharides all share the common feature of being comprised of two glucose molecules joined by an α -glycosidic bond (Fig. 1a). The five α -diglucosides are trehalose, kojibiose, nigerose, maltose, and isomaltose, and these sugars differ only in the position of their glycosidic bond. These disaccharides can serve as a source of nutrition for humans and are found in a wide variety of mushrooms, grains, fruits, vegetables, and more recently processed foods and beverages (Da Costa Leite et al. 2000; Elbein et al. 2003; Gerschenson et al. 2017). For a discussion of human disaccharide metabolism, several recent reviews have been published that explore this topic in detail (Rose et al. 2018; Kluch et al. 2020). Perhaps not surprisingly, not all α -diglucosides have been studied equally, and an analysis of PubMed indexed articles in April 2020 found a disproportionate number of studies (over 11,000) of maltose metabolism (Fig. 1b). Furthermore, maltose metabolism has been summarized from a variety of contexts and there are a number of excellent reviews published (Van Der Maarel et al. 2002; Ajita and Thirupathihalli 2014; Lentze 2018), and therefore we have excluded it from this review. Trehalose is the second most studied α -diglucoside and also has been reviewed (Elbein et al. 2003; Avonce et al. 2006; Ruhel et al. 2013), however we have included it in this review as several reports have been recently published that shed new light on bacterial trehalose degradation. For the remaining three α -diglucosides, there has not been a robust summary of past research concerning bacterial metabolism or discussion of emerging research areas. Consequently, the purpose of this review is to encapsulate the current understanding of the

diversity of bacterial α -diglucoside metabolism and highlighting critical structural features that affect enzyme activity when a substrate is one of the uncommon α -diglucosides trehalose, kojibiose, nigerose, or isomaltose. The enzymes included for discussion were chosen based on the availability of physiological (*in vivo*) data or biochemical (*in vitro*) data that went beyond standard kinetic characterization. We conclude the review with a roadmap for the fundamental questions that still remain to be answered for bacterial α -diglucoside metabolism, specifically regarding advancing biotechnology and biomedicine.

TREHALOSE (α -1,1- α DIGLUCOSIDE)

Biological Functions

Bacteria most commonly use trehalose as a stress protectant (Argüelles 2000; Elbein et al. 2003; Avonce et al. 2006). A variety of studies in *E. coli* have shown trehalose accumulation under heat, cold, osmotic, and other abiotic stress factors (Newman et al. 1993; Argüelles 2000; Iordachescu and Imai 2008). There are three modes of action believed to confer the protectant activity; chemical stability, water replacement, and sugar glass formation (Iturriaga et al. 2009; Jain and Roy 2009; Luyckx and Baudouin 2011). Trehalose is a non-reducing diglucoside, which makes it incapable of forming reactive aldehyde or ketone groups and its glycosidic linkage is relatively low energy (1 kcal/mol compared to the 27 kcal/mol glycosidic bond of sucrose) (Ohtake and Wang 2011). Furthermore, trehalose lacks internal hydrogen bonds, which facilitates a water replacement mode of stress protection (Brown et al. 1972). Specifically, the ability to form numerous hydrogen bonds with phospholipids and membrane proteins increases their stability during osmotic and thermal stress (Leslie et al. 1995; Leidy et al. 2004; Choi et al. 2006; Albertorio et al. 2007). These stabilized hydrogen-bond interactions are additionally beneficial as trehalose enters its glassy state, allowing an amorphous solid to surround, stabilize, and protect other macromolecules upon desiccation or high temperatures (Roos 1993; Miller et al. 1997; Liu et al. 1997).

In addition to stress mitigation, certain species of bacteria also use trehalose as a structural molecule or for energy storage. The *Mycobacterium* and *Corynebacterium* families possess members which incorporate modified trehalose in the form of trehalose dimycolate or trehalose monomycolate into their membrane glycolipids, making it a popular target for drug development in treating tuberculosis or diphtheria (De Smet et al. 2000; Nobre et al. 2014). Trehalose interactions with membrane phospholipids and membrane proteins during freeze-drying allow for the stabilization of these molecules, thereby increasing survival of frozen cells (Leslie et al. 1995). As carbon and energy storage molecule, *Streptomyces griseus* has been observed to accumulate 25% trehalose by dry weight under glucose abundant conditions, but as low as 1% under glucose limiting conditions, suggesting that glucose is converted to trehalose in carbohydrate abundant conditions (McBride and Ensign 1987). Conversely, *S. griseus* spores accumulate trehalose when grown in nutrient limiting conditions. The concentration of trehalose varies from 2% to 20% depending upon whether cells were grown in liquid or solid media with previous exposure to nutrient limiting conditions (Kendrick and Ensign 1983; McBride and Ensign 1987). Spores with larger quantities of trehalose had increased heat and desiccation resistance but a reduced

germination rate, suggesting a trade-off between spore survival and ease of departure from a dormant state (McBride and Ensign 1987).

Finally, trehalose serves as a carbon and energy source for many bacteria, and exploiting trehalose metabolism has increasingly been of interest for modulating the composition of the human gut microbiome. For example, trehalose has recently been studied as a potential prebiotic, as it selectively promotes the growth of beneficial *Bifidobacterium* and their production of bacteriocins (Sanz et al. 2005; Chen et al. 2007). Conversely, an epidemiological study indicated that higher trehalose consumption correlated with increased virulence of *Clostridium difficile* (Collins et al. 2018). Conflicting arguments also present themselves in terms of oral pathogens, with some studies indicating increased growth of the common oral pathogen *Streptococcus mutans* while others note decreased growth in relation to trehalose consumption (Neta et al. 2000; Hodoniczky et al. 2012a).

Biosynthesis

There are five potential biosynthetic pathways to generate trehalose in bacteria. The first pathway discovered, and arguably the most widely distributed, is direct synthesis of trehalose from monosaccharides (Cabib and Leloir 1958). This reaction involves the combination of glucose 6-phosphate and UDP-glucose to form trehalose 6-phosphate, which is performed by trehalose 6-phosphate synthase (TPS; E.C. 2.4.1.15). Subsequently, Tre-6-P is dephosphorylated by trehalose-phosphatase (TP; E.C. 3.1.3.12) (Elbein et al. 2003). First identified in *S. cerevisiae*, it has since been characterized in several bacterial species such as *E. coli*, *Mycobacterium*, and *Xanthomonas*, as well as archaea, plants, and fungi (Elbein et al. 2003; Avonce et al. 2006; Carroll et al. 2007; Paul et al. 2008).

The next most prevalent pathway converts maltoligosaccharides into trehalose, which is also the preferred industrial method to generate this diglucoside (Song et al. 2016; Cai et al. 2018). The first step involves maltooligosyltrehalose synthase (E.C. 5.4.99.15) converting the reducing end of a maltooligosaccharide from an α -1,4 bond to an α -1,1 bond. Next, trehalose is cleaved from the oligosaccharide by maltooligosyltrehalose trehalohydrolase (E.C. 3.2.1.141) (Song et al. 2016). As maltooligosaccharides are an integral reactant, organisms possessing this pathway are typically starch producing or starch consuming. Initially identified in *Arthrobacter*, this two-step generation of trehalose is known as either the MTSase MTHase pathway or the TreY – TreZ pathway (Nakada et al. 1955; Elbein et al. 2003). A pBLAST genome analysis performed in 2006 indicated that approximately 50% of bacteria and 10% of archaea possess this pathway (Avonce et al. 2006).

The remaining three biosynthetic pathways employ single-step conversion mechanisms, however they are less prevalent within trehalose producing organisms and not used for commercial production of trehalose. The TreP (E.C. 2.4.1.64) pathway has been shown to use glucose 1-phosphate and glucose to generate trehalose and inorganic phosphate *in vitro*, specifically under low phosphate concentrations (Wannet et al. 1998). This pathway was initially identified in fungi, but has since been observed in few bacteria, such as *Thermoanaerobacter tengcongensis* (Wannet et al. 1998; Elbein et al. 2003; Avonce et al. 2006). The trehalose glycosyltransferring synthase (TreT; E.C. 2.4.1.245) pathway is capable of generating trehalose from ADP-glucose and glucose, and has been observed in

both archaea and bacteria (Qu et al. 2004; Avonce et al. 2006; Nobre et al. 2008). The final pathway; trehalose synthase, has only been observed within bacteria. Similar to the TreY-TreZ pathway, it converts α -1,4 bonds to α -1,1 bonds, however, it is highly specific for maltose (Nishimoto et al. 1996; Avonce et al. 2006).

Degradation and Metabolism

When used as a nutrient source by bacteria trehalose can be cleaved during transport into the cell or by the action of extracellular or cytoplasmic enzymes. Trehalose transported through the phosphotransferase system (PTS) is cleaved by trehalose-6-phosphate hydrolases (E.C. 3.2.1.93), and generates glucose and glucose 6-phosphate for entry into metabolism (Fig. 2a) (Elbein et al. 2003; Ruhel et al. 2013). The PTS systems of a diverse range of bacteria have been reviewed elsewhere (Kotrba et al. 2001; Saier 2015), therefore we will focus here on non-membrane associated trehalases.

Soluble trehalases (E.C. 3.2.1.28) split trehalose to produce two glucose molecules. As classified by the Carbohydrate Active enZyme (CAZyme) database, trehalases can belong to glycoside hydrolase (GH) families 15, 37, and 65 (Lombard et al. 2014). Enzymes belonging to the GH15 and GH65 families have been characterized as cleaving a diverse range of substrates, including trehalose (Lombard et al. 2014; Sakaguchi 2020). In this review, we will only discuss the GH37 enzymes, as this family has exclusively trehalase activity. Trehalases of the GH37 family have been identified in species of 178 genera (Lombard et al. 2014), and suggests that despite trehalose being relatively low in abundance in the environment it is still a metabolite with the potential to provide a competitive edge for those organisms capable of consuming it.

Biochemical characterization of GH37 trehalases has suggested that these enzymes are largely comparable despite being made by diverse bacteria. Bacterial trehalases from a broad range of species have been physiologically, biochemically, or structurally characterized and representative examples include *E. coli* TreA and TreF, *E. cloacae* TreA, *N. punctiforme* TreH, *R. marinus* TreH, *C. japonicus* Tre37A, *X. citri* sp. *citri* TreA, and *Zunongwangia* sp. TreZ (Table 1) (Boos et al. 1987; Horlacher et al. 1996; Uhland et al. 2000; Gibson et al. 2007; Jorge et al. 2007; Yoshida and Sakamoto 2009; Cheng et al. 2016; Adhav et al. 2019). Summarized below are several key points from the characterization of these GH37 enzymes, with a particular emphasis on the structural features important for catalysis.

The structure of the *E. coli* trehalase TreA in complex with competitive inhibitors validoxylamine A and 1-thiatrehazolin provided insight into the GH37 catalytic mechanism (Gibson et al. 2007). This periplasmic enzyme contains a diagnostic $(\alpha/\alpha)_6$ barrel structure characteristic of many α -glycosidases. X-ray crystallography of the enzyme complexed with competitive inhibitor indicated that residues Asp312 and Glu496 act as the catalytic acid and base, respectively. Additionally, the pseudo sugar ring present in validoxylamine A and the glucose moiety present in 1-thiatrehazolin exposed the presence of a large hydrophilic binding platform in the enzyme. The positioning of the +1 and -1 subsites relative to the competitive inhibitors suggested that significant conformational changes are required during substrate binding and cleavage due to the presence of an expansive binding pocket.

Mutational analysis of the cytoplasmic *E. coli* TreF identified two residues, Thr172 and Ala82, which when mutated increased the catalytic activity of the enzyme six-fold (Uhland et al. 2000). Amino acid sequence analysis by Sakaguchi *et al.* suggested that these are not conserved residues, but Thr172 appeared to be located within a sequence motif that is likely surrounded by tryptophan residues involved in the hydrophilic binding platform (Gibson et al. 2007; Adhav et al. 2019; Sakaguchi 2020). It was argued that a mutation in this region may affect the surrounding hydrophilic binding platform and can generate a more accessible structure with an increased rate of binding. It was less clear why the second mutation, Ala82Thr, was increased TreF activity, as this residue was not located within a conserved region.

Subsequently, two structures of the periplasmic *E. cloacae* TreA (PxEcTre) were solved, one in complex with validoxylamine A and one without any substrates (Gibson et al. 2007; Adhav et al. 2019). As observed with the *E. coli* TreA, the *E. cloacae* GH37 enzyme had an (α/α)₆ barrel structure and the positioning of the acid and base acting residues, Asp312 and Glu496, were also similar. Interestingly, the structure solved without substrate identified a lid loop that helped explain the large conformational changes observed when substrate was bound, as well as a side loop that further stabilized substrate binding. Furthermore, mutational analysis found a critical non-catalytic residue (Trp447) that was required for proper binding pocket formation. A second non-catalytic residue that was found to be important for activity but not enzyme stability was Glu279, which was predicted form a salt bridge. Both of these residues are conserved in several bacterial trehalases.

Studies of *Zunongwangia* sp. TreZ utilized site-directed mutagenesis and error-prone-PCR to generate mutants with increased catalytic efficiency (Cheng et al. 2016). Two mutations, Tyr227His and Arg442Gly, were observed to act both individually and synergistically to increase the substrate specificity and reaction rate of TreZ. The authors argued that these mutations improved activity via expansion of the substrate binding pocket. The presence of both mutations resulted in a catalytic efficiency three-fold greater than that of the wild-type protein. Interestingly, Tyr227 appears to be conserved amongst characterized trehalases, while Arg442 is not.

The saprophytic soil bacterium *Cellvibrio japonicus* possesses two periplasmic GH37 trehalases, Tre37A and Tre37B (Garcia et al. 2020). Growth curve analysis indicated that only one of these, Tre37A, is required for trehalose utilization. This agreed with biochemical data, which found that Tre37A was a potent trehalase while Tre37B had no detectable activity (Table 1). Amino acid alignment of the *C. japonicus* trehalases alongside other characterized trehalases found that they possessed the conserved catalytic domains, signature motifs, and hydrophilic bonding platform identified from *E. coli* TreA (Gibson et al. 2007; Garcia et al. 2020).

KOJIBIOSE (α -1,2 DIGLUCOSIDE)

Biological Functions

Initially isolated in 1953 from sake, kojibiose is a reducing sugar is also found in beer, honey, caramelized glucose, and as a byproduct of dextran formation (Aso and Shibasaki

1953; Watanabe and Aso 1960; Matsuda et al. 1961). Kojibiose is classified as a rare sugar because it occurs infrequently in nature and in small quantities (Granström et al. 2004; Beerens et al. 2017). For example, honey contains approximately 0.5 g to 1.5 g kojibiose for every 100 g total mass, depending upon the origin of the honey (Schievano et al. 2017). Currently, the only known role for kojibiose in nature is as an energy source used for its glucose moieties. Regardless of its availability, kojibiose has garnered increased interest as a potential prebiotic and for its role as an α -glucosidase inhibitor (Ogawa et al. 1998; Sanz et al. 2005). Single culture studies of human gut microbiome indicate that *Bifidobacterium*, *Lactobacillus*, *Clostridium*, *Bacteroides*, and *Eubacterium* can grow using kojibiose as a sole carbon source (Chaen et al. 2001; Nakada et al. 2003; Gibson et al. 2004). Studies with bacterial consortia from fecal material suggested that kojibiose had a high selectivity for *Bifidobacterium* and resulted in greater acetic acid yields than many other disaccharides tested (Sanz et al. 2005).

Kojibiose can act as an α -glucosidase inhibitor, and is being explored for the treatment for different diseases. For example, drug development to treat HIV infections is testing kojibiose, as the virus is reliant on α -glucosidases to produce the glycoproteins that interact with host CD4 cells during virus reproduction (Kwong et al. 1998; Ogawa et al. 1998). Kojibiose alone behaves as a weak competitive inhibitor for α -glucosidase, but kojibiose pseudodisaccharides, which are saccharides possessing five membered rings where oxygen is present in the 1- and 3- position, have been discovered with increased potency (Ogawa et al. 1998; Barker and Rose 2013). Kojibiose is also being explored as a treatment for diabetes, as inhibiting α -glucosidase activity would reduce carbohydrate metabolism and glucose absorbance in the human gut (Phung et al. 2010).

Synthesis

Early attempts to isolate kojibiose from natural sources returned incredibly low quantities of the desired sugar (Watanabe and Aso 1959; Matsuda et al. 1961). Although honey was observed to contain approximately 0.5% - 1.5% kojibiose by weight, purification from this source had a return rate much less than 0.001% and required numerous concentration and purification steps, likely due in part to the wide variety of carbohydrates present within honey (Watanabe and Aso 1959). The low natural abundance of kojibiose, coupled with the poor recovery from natural sources, has led to increased interest in the chemical production of this α -glucoside. One described method involves the acetolysis of dextrans utilizing a mixture of acetic anhydride and sulfuric acid (Matsuda et al. 1961; Duke et al. 1973). The source of dextrans typically comes from *Leuconostoc mesenteroides* NRRL B-1299, which forms higher than average proportions of α -1,2 glycosidic linkages than other species. While this method is able to produce kojibiose from dextrans, the products require intensive purification that renders it not industrially viable. An alternative chemical method employed branched glucopyranose and zinc chloride in a modified Koenigs-Knorr reaction. This method generates a mixture of glucose, kojibiose, and the β -diglucoside sophorose, and as with previous attempts to chemically produce kojibiose resulted in low yields (McGrath et al. 1969).

Biosynthesis of kojibiose has also been successful, specifically a four-step reaction can generate kojibiose with a 38% yield and 99% purity (Diez-Munico et al. 2014). A mixture of sucrose and lactose is initially incubated with dextransucrase from *L. mesenteroides* to form 4'-galactosyl-kojibiose and fructose. A *S. cerevisiae* strain then metabolizes excess fructose and sucrose from the reaction mixture prior to incubation with β -galactosidase from *K. lactis*. The β -galactosidase cleaves 4'-galactosyl-kojibiose to release kojibiose and galactose (see Fig. 2 from Diez-Munico et al. 2014). The kojibiose is purified with liquid chromatography and while this method can generate large quantities of purified kojibiose, as outlined above it requires multiple steps and extensive processing to remove microbiological contaminants and undesirable side products.

A more efficient *in vitro* method of kojibiose production employs a single sucrose phosphorylase obtained from *B. adolescentis* (Verhaeghe et al. 2016). This method produces maltose and kojibiose by transglycosylation of glucose and sucrose (see Scheme 1 from Verhaeghe et al., 2016). While the wild-type enzyme preferentially forms maltose, introduction of L314I and Q345S mutations into the active site shifted production towards kojibiose. Incubation of the reaction mixture with *S. cerevisiae* was sufficient for removal of undesirable side products and resulted in a kojibiose purity of 99.5%. Additionally, the modified enzyme appeared to maintain a high rate of activity throughout a one-week incubation period at 55 °C, which would allow it to be used for multiple production cycles.

Degradation and Metabolism

Phosphorylases are transferase enzymes that catalyze the reversible transfer of phosphates, which allows for both synthesis and degradation to be mediated by a single enzyme. Bacterial production and degradation of kojibiose is dependent upon one of two enzyme classes; kojibiose phosphorylase (E.C. 2.4.1.230, GH65) (Fig. 2b) or sucrose phosphorylase (E.C. 2.4.1.7, GH13_18), though sucrose phosphorylase activity on kojibiose is considered an exclusively *in vitro* non-specific side reaction. As kojibiose phosphorylases are natively more specific than sucrose phosphorylases for the production and degradation of kojibiose, this review will focus on kojibiose phosphorylases. For a review on sucrose phosphorylases, two reviews provide an excellent background on those enzymes (Puchart 2015; Franceus and Desmet 2020). Here we will limit our discussion to the GH65 enzymes, with particular emphasis on the structural features important for catalysis.

Bacterial kojibiose phosphorylases degrade kojibiose to D-glucose and β -D-glucopyranose 1-phosphate *in vivo*, but have also been shown to produce kojibiose *in vitro* when certain equilibrium conditions are favored (Chaen et al. 1999; Chaen et al. 2001; Yamamoto et al. 2006; Okada et al. 2014). The GH65 family, and therefore potential kojibiose phosphorylase activity, is widely distributed amongst Gram-positive bacteria, and examples can be found in *Bacillus*, *Clostridioides*, *Enterobacter*, and *Lactobacillus* genera (Lombard et al. 2014). However, the GH65 family also has members with α -trehalase, maltose phosphorylase, and nigerose phosphorylase activities, therefore without experimental data a definitive assessment of a GH65 enzyme can be difficult based on sequence similarity alone. Therefore, we will limit our discussion of kojibiose phosphorylases to those that have been biochemically characterized, and include enzymes from *Thermoanaerobacter brockii*,

Calicellulosiruptor saccharolyticus, and *Escherichia coli* (Chaen et al. 1999; Chaen et al. 2001; Yamamoto et al. 2006; Okada et al. 2014).

The first kojibiose phosphorylase identified belonged to thermophilic spore forming bacterium *T. Brockii* ATCC 35047, and was designated KojP (Chaen et al. 1999). This enzyme was able to both synthesize and degrade kojibiose, though it showed a greater K_{CAT} during kojibiose degradation. Additionally, the enzyme showed greater specificity for kojibiose degradation compared to the degradation of other disaccharides. This is in direct contrast to the synthesis reaction, which produced a wide array of products, including kojibiose but also minor amounts of trehalose, sorbose, nigerose, maltose, and isomaltose when using different acceptor substrates. These minor products suggested that *T. Brockii* KojP may have broad specificity for the formation of α -1,2 glycosidic bonds (Chaen et al. 1999). Site-directed mutagenesis studies of *T. Brockii* KojP found that Asp362Asn, Lys612Gln, or Glu642Gln changes abolished kojibiose degradative capabilities. In contrast, the mutation of Asp459Asn resulted in a near seven-fold increase in K_M for kojibiose and glucose substrates. This mutation likely affected the active site in such a way as to eliminate phosphorolysis, while maintaining transglycosylation and even increase specificity for these two saccharides (Yamamoto et al. 2004). These residues are conserved among other characterized kojibiose phosphorylases, with Asp362 acting as part of the hydrophilic substrate-bonding site, and Lys612 and Glu642 being hydrophilic bonding residues. Additionally, the Asp459 residue critical for kojibiose degradation is located near a loop region identified as being critical for kojibiose phosphorylation in *C. saccharolyticus* (Okada et al. 2014). Mutation of these residues in other kojibiose phosphorylases may produce similar increases in kojibiose formation as well as decreased kojibiose phosphorylation. A separate study generated a *T. Brockii* kojibiose phosphorylase (KojP) and trehalose phosphorylase (TreP) chimera and observed a shifted ratio in favor of kojibiose production, however, the overall rate of the reaction was reduced (Yamamoto et al. 2006). Together, these studies identified the critical elements that alter the regioselectivity of *T. Brockii* KojP, which will be important to ensure a high selectivity for kojibiose formation if used in selective *in vitro* biotechnology applications.

The study of a kojibiose phosphorylase from the anaerobe *Calicellulosiruptor saccharolyticus* provided a detailed crystal structure (Yamamoto et al. 2011; Okada et al. 2014). Biochemical characterization of the *C. saccharolyticus* kojibiose phosphorylase (Cs-KP, locus ID: Csac_0439) indicated that the pH and temperature optima for kojibiose synthesis were distinct from the optima for kojibiose degradation (Table 2) (Yamamoto et al. 2011). Additionally, the specific activity for synthesis is six-fold greater than that of the degradation, further indicating that the production of kojibiose is favored and dependent on the concentration of reactants. Optimization of reaction conditions was not determined, as the syntheses also generated a variety of products and favored the production of kojitriose over kojibiose. Nevertheless, the dependency on pH and temperature to dictate the directionality of Cs-KP might be exploited industrially towards kojibiose synthesis.

Crystal structures of Cs-KP in complex with either glucose and phosphate or kojibiose and sulfate identified an extended loop region critical in forming the active site and several residues involved in substrate interactions (Okada et al. 2014). As is characteristic for many

GH65 enzymes, a single acidic catalytic residue (Glu483) was observed in addition to a molecule of phosphate maintained in the correct orientation by three amino acid residues for a nucleophilic attack. The structures indicated that both glucose substrates and kojibiose substrates are complexed in similar conformations, with several conserved hydrophilic residues stabilizing the substrate at the +1 and -1 subsites. Site-directed mutagenesis identified Glu392 as being critical for kojibiose degradation, though mutation of residues Trp391 and Thr417 also reduced kojibiose catalysis. The Trp391 and Glu392 residues precede the critical loop region and are conserved amongst kojibiose phosphorylases. Interestingly, the less-impactful Thr417 residue is located within the loop region, critical for substrate interaction, and shares identity with the highly active *T. brockii* KojP but not the lesser active *E. coli* YcjT. Although the loop structure was identified as being crucial for kojibiose interaction, it shares reduced identity in comparison to other kojibiose phosphorylase regions.

Recently, an *E. coli* kojibiose phosphorylase (YcjT) was identified and characterized (Mukherjee et al. 2018). A gene cluster of unknown function was observed in *E. coli*, containing an assortment of putative transporters in addition to two putative hydrolases/ phosphorylases that shared homology with sucrose phosphorylases from *Bifidobacterium* (Sprogøe et al. 2004; Mukherjee et al. 2018). However, YcjT showed only moderate identity with *T. brockii* KojP (30.4%) and Cs-KP (32.2%). Kojibiose synthesis activity was observed in solutions containing 8.0 mM β -D-glucose-1-phosphate, while degradation was observed in solutions containing 5 mM phosphate (Mukherjee et al. 2018). Examination of the amino acid sequence indicated the presence of a Ser409Thr mutation, deviating from the conserved hydrophilic bonding template and loop region identified from *C. saccharolyticus* kojibiose phosphorylase (Okada et al. 2014; Mukherjee et al. 2018). Interestingly, while quantifiable enzyme activity was observed, *E. coli* proved incapable of growth on media containing kojibiose as the sole carbon source. Consequently, it is currently unknown what physiological role YcjT plays in *E. coli* metabolism.

NIGEROSE (α -1,3 DIGLUCOSIDE)

Biological Functions

Nigerose, also known as sakebiose, is a reducing sugar originally isolated from the degradation of nigeran, a polysaccharide found in *Aspergillus niger* (Konishi and Shindo 1997; Ma et al. 2019). As the synonym suggests, this α -glucoside can also be found in sake, as well as beer, honey, as a product of glucose caramelization and as a byproduct of dextran degradation (Aso and Shibasaki 1953; Matsuda et al. 1961; Konishi and Shindo 1997; Ma et al. 2019). NMR quantification of the carbohydrates found in honey observed 0.23 g to 0.65 g nigerose in 100 g honey, though the concentration varied depending upon origin (Schievano et al. 2017). Additionally, nigerose has also been found in the red algae *Dilsea edulis*, albeit in small quantities (Peat et al. 1957). Consequently, nigerose is often considered an environmentally rare sugar, as it occurs in few organisms and in small quantities (Granström et al. 2004; Kraus et al. 2016).

Nigerose has been evaluated for its potential as a prebiotic, alternative sweetener, cryoprotectant, immunoprotectant, and possible mechanism for drug delivery (Murosaki et

al. 1999; Sanz et al. 2005; Al-Otaibi et al. 2018). This α -glucoside has high potential for use as a reduced calorie sweetener (Hodoniczky et al. 2012a; Hodoniczky et al. 2012b). It is resistant to degradation by the common human oral pathogen, *Streptococcus mutans*, which is an advantage over other sweeteners such as glucose, trehalose, and sucrose (Hodoniczky et al. 2012a). In addition to its potential as a sweetener and oral prebiotic, there is some evidence for its potential as a digestive prebiotic. Inoculation of complex fecal cultures with nigerose proved to be somewhat selective for the growth of *Bifidobacterium* and *Bacteroides* and reduced the growth of *Clostridium*, however, these trends were more pronounced when kojibiose and sophorose were evaluated (Sanz et al. 2005). Increased resistance to *Lactobacillus plantarum* infection was also observed when mice were given oral doses of nigerose and mixed nigerans, further identifying nigerose as a potential therapeutic for infectious disease (Murosaki et al. 1999). The addition of nigerose to human cell lines prior to cryopreservation resulted in increased viability after thawing, which suggests an industrial application if more widely adopted (Al-Otaibi et al. 2018).

Biosynthesis

Nigerose research has primarily concentrated on production of this α -glucoside, with *in vitro* methods developed to degrade dextrans with nigerose phosphorylases or sucrose phosphorylases (Robyt 2008; Nihira et al. 2014; Franceus et al. 2019). Recent reports have identified and characterized few phosphorylases able to generate nigerose with and without extensive mutagenesis (Nihira et al. 2012; Nihira et al. 2014; Kraus et al. 2016; Franceus et al. 2019). While phosphorylase reactions are typically reversible, rational design of enzymes and careful control of conditions are able to force the nigerose forming reaction (Nihira et al. 2014). In this review, we will summarize the current knowledge concerning bacterial nigerose phosphorylases (GH65; E.C. 2.4.1.297) (Fig. 2c) and a nigerose-active sucrose phosphorylase mutant (CH13_18; E.C. 2.4.1.7) from *Bacillus subtilis* (Table 3) (Kitao et al. 1994; Stam et al. 2006; Kraus et al. 2016).

At the time of this review the only characterized bacterial nigerose phosphorylase (locus ID: *Cphy1874*) that has been characterized is from *Lachnoclostridium phytofermentans*, previously known as *Clostridium phytofermentans* (Nihira et al. 2012; Yutin and Galperin 2013). *Cphy1874* was observed to weakly degrade kojibiose (<1%), and produce small quantities after long periods of incubation. Conversely, shorter periods of incubation resulted in a high level of specificity for reactions involving nigerose. Nigerose production was observed when utilizing glucose, however, nigerose analogues were produced when a wide variety of substrates such as, galactose, xylose, and 1,5-anhydro-D-glucitol were used as acceptors (Nihira et al. 2012). NMR spectra analysis of products formed by incubation with glucose indicated that α -1,3 glucosides were the major product formed. Some transglycosylation of nigerose into kojibiose was observed after extended periods of incubation, which hinted that the ratio of glucose, nigerose, and kojibiose influences product formation (Nihira et al. 2014). The authors conclude that the specificity for both nigerose degradation and production over other α -glucosides by *Cphy1874* might be desirable for industrial nigerose production. A subsequent study using *Cphy1874* successfully produced nigerose from sucrose using a one-pot reaction with three additional enzymes (see Figure 3A from Nihira et al., 2014) (Nihira et al. 2014; Franceus and Desmet 2020). Briefly, a sucrose

phosphorylase from *Bifidobacterium longum* JCM1217 (locus ID: BLLJ_0106) used phosphate to cleave sucrose and yielded α -D-glucose-1-phosphate that was subsequently converted into α -D-glucose-6-phosphate by α -phosphoglucomutase from *Thermococcus kodakarensis* KOD1 (locus ID: TK1108). The α -D-glucose-6-phosphate was then converted to β -D-glucose-1-phosphate by β -phosphoglucomutase from *Lactococcus* sp. (Sigma-Aldrich; product ID P4109). The final step in the one-pot reaction had *Cphy1874* combine β -D-glucose-1-phosphate and D-glucose to yield nigerose and inorganic phosphate. The phosphate would then allow for sucrose phosphorylase to continue the initial reaction. When incubated for 100 hours approximately 67% reaction yield of nigerose was obtained. The reaction schema allowed for utilization of all side products formed and mitigated nigerose degradation as substrates were continually being produced while limiting phosphate to either the sucrose phosphorylase or the nigerose phosphorylase reactions (Nihira et al. 2014).

Broad-range sucrose phosphorylases are another potential source of nigerose production. One such sucrose phosphorylase from *Leuconostoc mesenteroides*, *LmSPase*, was observed to form both kojibiose and nigerose upon incubation with sucrose and D-glucose, however, kojibiose production was more than double that of nigerose; 7.5% and 3.2% w:v reaction mixture respectively (Kitao et al. 1994). Increasing the reaction time produced no significant change in product concentration. Similar reactions were observed when the initial reactants consisted of solely sucrose, α -D-glucose-1-phosphate and D-glucose, or α -D-glucose-1-phosphate and sucrose, although the products were greatly reduced in these reactions.

The limited success using *LmSPase* to generate nigerose was enough that other sucrose phosphorylases were also characterized and included one from *Bifidobacterium adolescentis* (*BaSP*) (Franceus et al. 2019; Franceus and Desmet 2020). Initial studies of *BaSP* activity noted a strong preference for the formation of kojibiose or maltose, but no significant production of nigerose (Kraus et al. 2016). However, a Gln345Phe mutation of the loop region affecting binding of acceptor and donor substrates resulted in production of nigerose and maltose in equal concentrations. Addition of DMSO to 40% concentration increased nigerose production six-fold and further decreases the concentration of maltose produced. Introducing two additional mutations to this loop region, Asp342Gly and Tyr344Gln, resulted in a nine-fold increase in relative activity and five-fold increase in nigerose:maltose production over that of the single mutation. Addition of a fourth mutation to the acceptor site loop, Arg135Tyr, further increased the relative activity by 36-fold and the nigerose:nmaltose production by seven-fold (Franceus et al. 2019; Franceus and Desmet 2020). When the quadruple mutant was tested for nigerose production at a larger scale it was observed to have a 43% reaction yield (Franceus et al. 2019).

Degradation and Metabolism

Due to its low natural abundance and high cost to produce at an industrial scale, nigerose metabolism has focused on synthesis and not degradation. While the degradation of this α -glucoside has received less attention, two enzymes with nigerose hydrolytic activities have been observed, one from *E. coli* (YgjK) and one from *Lactobacillus johnsonii* (*LjAg31*, locus ID: LJ_0569) (Fig. 2d) (Kurakata et al. 2008; Kang et al. 2009; Miyazaki et al. 2013). Interestingly, *E. coli* YgjK is classified as a GH63 enzyme (E.C. 3.2.1.20 and E.C. 3.2.1.84),

and members of the GH63 family are exo-acting α -glucosidases with characterized prokaryotic GH63 enzymes functioning primarily as mannosylglycerate hydrolases (Lombard et al. 2014). The *L. johnsonii* enzyme *LjAg31* is classified as a GH31 enzyme (E.C. 3.2.1.84), a family which possess a broad range of activities, including; α -glucosidase, α -galactosidase, α -mannosidase, α -xylosidase, among others. Other enzymes with the classification of E.C. 3.2.1.84 are capable of hydrolyzing α -1,3 glycosidic linkages, however these enzymes are typically exo-acting and cleave at the reducing end of α -1,3 glucans to produce glucose (Zonneveld 1972; Ait-lahsen et al. 2001). We have summarized below the work done to date on the YgjK and *LjAg31* enzymes.

Thus far *E. coli* possesses the only enzyme currently characterized as a GH63 nigerose hydrolase, YgjK (Lombard et al. 2014). An NCBI BlastP search of the amino acid sequence of this enzyme found homologs within *Enterobacter*, *Vibrionaceae*, and *Alteromonas* orders (matrix BLOSUM62, expect threshold 1×10^{-5}). Although the identification of homologous in this manner initially seems promising, it is likely that these sequences will need to be analyzed individually for structural similarities to YgjK. The solved structure of *E. coli* YgjK showed two distinct domains and a cleaved N-terminus, which indicated secretion into the periplasm or extracellular matrix (Tonozuka et al. 2004; Kurakata et al. 2008). The proposed catalytic residues are Asp501 and Glu727 acting as the acid and base, respectively (Barker and Rose 2013). These residues are not conserved among the other characterized nigerose-acting enzymes. It was also found that YgjK bound glucose, mannose, and galactose (Barker and Rose 2013). This result agrees with biochemical data that YgjK can hydrolyze trehalose, maltooligosaccharides, and kojibiose, however the enzyme displayed a strong preference for α -1,3 glycosidic linkages (Table 3) (Kurakata et al. 2008). The overall low affinity for nigerose suggests that it is likely not the preferred substrate of YgjK. This is corroborated by the inability of *E. coli* to grow using nigerose as a sole carbon source. Mutation of the catalytic Glu727Ala or the binding pocket residue Asp324Asn converted YgjK into a glucosynthase, producing 2-O- α -D-glucopyranosyl-D-galactopyranose from galactose and β -D-glucofuranose and no longer had any nigerose hydrolase activity (Miyazaki et al. 2013). These mutational studies of YgjK highlight the versatility and promise of GH63 nigerose hydrolases to produce high-value oligosaccharides for biotechnology and biomedicine.

The second enzyme with nigerose hydrolase activity was obtained from *L. johnsonii* (Kang et al. 2009). The cytoplasmic *L. johnsonii* *LjAg31* enzyme shows activity against a broad range of substrates including maltose, isomaltose, kojibiose, and nigerose. Despite being able to cleave several authentic α -diglucoside substrates, *LjGH31* had the greatest affinity for the artificial substrate pNPG. This is interesting as the majority of characterized GH31 enzymes exhibit α -glucosidase activities while this enzyme shows a high affinity for transglucosylation, forming both pNP α -nigeroside and pNP α -isomaltoside when incubated with pNP α -maltoside (Kang et al. 2009; Lombard et al. 2014). The authors proposed that the *LjAg31* enzyme warranted classification as an α -1,3 glucosidase due to the K_{CAT}/K_M value for nigerose being the largest observed amongst all substrates (Kang et al. 2009). If the substrate specificities and catalytic efficiencies are compared between the two nigerose hydrolase enzymes of *E. coli* and *L. johnsonii*, it is observed that *LjAg31* has a 128-fold higher affinity, but a similar catalytic rate for nigerose (Kurakata et al. 2008; Kang et al.

2009). An in-depth sequence comparison of these two hydrolytic enzymes may provide insight as to substrate specificity and catalytic efficiency, however this may prove difficult as the two enzymes share low identity (~15%).

ISOMALTOSE (α -1,6 DIGLUCOSIDE)

Biological Functions

Isomaltose was initially described as a reducing, non-fermentable sugar produced by the condensation of glucose, and as its name suggests, has historically been described as an isomer of maltose (Wolfrom et al. 1949; Bacon and Bacon 1954). When isolated from starch, isomaltose was described simply as an anomalous branching unit serving to generate cross-linked chains of maltose (Meyer and Bernfeld 1940; Tsuchiya et al. 1949). Later studies stripped away α -1,4 maltose units from amylopectin and uncovered that cross-linking branch points consisted of α -1,6 isomaltose (Montgomery et al. 1949; Tsuchiya et al. 1949).

Isomaltose has been studied as an alternative sweetener, prebiotic, potential therapeutic for health issues such as constipation and similar digestive problems, a potential marker for macular degeneration, and for increased conversion efficiency of oligosaccharides containing α -1,6 mixed linkages (Sanz et al. 2005; Harrion et al. 2007; Yen et al. 2011; Abe et al. 2017; Luo et al. 2017; Fatoki et al. 2018). The human gut has only slight capacity for isomaltose hydrolysis and absorption, which allows this α -glucoside to have a reduced glycemic index when compared to more readily absorbed carbohydrates (Ang and Linn 2014). These health benefits are enhanced due to the prebiotic properties of isomaltose (Kohmoto et al. 1988). Early studies indicated that various strains of *Bifidobacterium* commonly found within the human gastrointestinal tract were capable of utilizing isomaltose. Studies using human fecal matter indicated that isomaltose had a small positive selective pressure on *Bifidobacterium*, *Bacteroides*, *Eubacterium*, and *Lactobacillus*, and a small negative selective pressure on *Clostridium* (Sanz et al. 2005). Incubation with isomaltose produced large quantities of acetic acid and minute quantities of lactic acid, propionic acid, and butyric acid, which are common metabolic byproducts of *Bifidobacterium* anaerobic metabolism (Sanz et al. 2005). As isomaltose is commonly found in foods such as sake, honey, and caramel, albeit in small quantities (Watanabe and Aso 1959; Watanabe and Aso 1960; Matsuda et al. 1961). For example, honey was determined to contain between 0.8 g and 4.75 g isomaltose per 100 g starting material, depending upon the origin of the honey, putting its concentration above that of both nigerose and kojibiose (Schievano et al. 2017). In addition to a primary function as a nutrient source, isomaltose has also been examined for its biomedical potential. A study involving a wide range of people in various states of health noticed that the concentration of isomaltose in urine strongly correlated with periods of fasting (Vitek et al. 1972). This may have potential in identifying populations with insufficient or unstable food sources who may need assistance. Furthermore, due to the low glycemic index of isomaltose it has been studied as an alternative sweetener for people with diabetes (Ang and Linn 2014). There is also some evidence that isomaltose has weak antimicrobial properties (Memarzadeh 2009; Fatoki et al. 2018). Specifically, a pharmaceutical involving chlorine mono- and di- halogenated

isomaltose has been patented as an oral mucosa cell permeation enhancer and low-grade antimicrobial (US Patent 2008/0171721 A1; 2008). These qualities in conjunction with its ubiquity in a variety of food and beverages potentially make isomaltose a convenient method of self-treatment before more potent antibiotics are required (Watanabe and Aso 1960).

Biosynthesis

Initially, isomaltose generation was an undesirable byproduct of starch degradation using α -amylases (Crabb and Shetty 1999). Common amylose starches are composed of primarily α -1,4 bound glucose, but up to 5% of the glycosidic bonds can be α -1,6 branch points (Pfister and Zeeman 2016). Typically, starch is degraded using α -amylases which cleave α -1,4 glycosidic bonds and release glucose, maltooligosaccharides, and α -1,6 isomaltose (Gopinath et al. 2017). Recently, isomaltose and isomaltooligosaccharides (IMOs) have gained interest as potential prebiotics and alternative sweeteners (Sanz et al. 2005; Harrion et al. 2007; Yen et al. 2011; Abe et al. 2017; Luo et al. 2017; Fatoki et al. 2018). IMO producing enzymes are largely described as glycoside hydrolases which also possess transferase activities (Lombard et al. 2014). Five such GH classes have been observed; GH13 (E.C. 3.2.1.133), GH31 (E.C. 3.2.1.20), GH57 (E.C. 2.4.1.18), GH66 (E.C. 3.2.1.11), and GH70 (E.C. 2.4.1.-) (Casa-Villegas et al. 2018). For a structural analysis of the enzymes involved in IMO synthesis, please refer to the following source (Casa-Villegas et al. 2018).

Commercially, GH31 transglycosylases from *Aspergillus* sp. are used due their strong preference towards generating isomaltose over other disaccharides, and also for bond synthesis rather than bond hydrolysis (Mangas-Sánchez and Adlercreutz 2015; Casa-Villegas et al. 2017). One transglycosylase from *A. nidulans* is capable of 50% conversion of maltose to other carbohydrate products (Kato et al. 2002; Goffin et al. 2010). Increasing the concentration of reactants is one proposed method for increasing conversion rates, while a second method involves combining enzymes (Viktor et al. 2013; Niu et al. 2017). Specifically, combining transglycosylating enzymes with amylases, glucoamylases or pullulanases reduces the formation of undesired byproducts and increases conversion to isomaltooligosaccharides (Viktor et al. 2013; Casa-Villegas et al. 2017; Niu et al. 2017).

Bacterial IMO generating enzymes have been found within each of the GH13, GH31, and GH70 classes, though the production of larger IMOs are favored over that of isomaltose (Lombard et al. 2014; Casa-Villegas et al. 2018). For that reason, here we will focus on the mutant Arg325Gln SmuA protein obtained from *Protaminobacter rubrum* (Lee et al. 2008). *P. rubrum* SmuA is classified as a GH13 sucrose isomerase, and the wild-type variant preferentially produces isomaltulose from sucrose, although a small shift towards producing isomaltose is observed when the reaction is supplemented with 5% glucose. Introduction of an Arg325Gln mutation within the conserved fructose-binding site present within sucrose isomerases shifted product yield away from isomaltulose and towards isomaltose. Isomaltose production was further increased when variant SmuA was incubated at 35°C and supplemented with 15% (w:v) glucose. Under these conditions, the relative activity for isomaltose production reached 46%. As few bacterial enzymes possess the required transglycosylation abilities, and fewer still preferentially produce isomaltose, future research may benefit from a rational engineering of bacterial sucrose isomerases in a similar manner.

Degradation and Metabolism

Many organisms possess transglycosylases capable of altering the α -1,6 bond present in starch and other dextrans to the more readily digested α -1,4 bond (Souza 2010). However, transglycosylation can generate products that do not have α -1,4 bonds, and several transglycosylases characterized thus far displayed low specific activity (Ajita and Thirupathihalli 2014; Mangas-Sánchez and Adlercreutz 2015). Alternatively, direct and efficient isomaltose hydrolysis can be performed by isomaltases (GH13_31; E.C.3.2.1.10), which are part of the oligo-1,6-glucosidase family (Fig. 2e, Table 4) (Lombard et al. 2014).

One of the first characterized oligo-1,6 glucosidases from *Bacillus* sp. F5 (MalL; gene ID: O16G_BACF5) was heterologously expressed in *E. coli*, purified, and the kinetic parameters determined for several potential substrates (Yamamoto and Horikoshi 1990). The recombinant enzyme had the greatest level of activity on panose, a trisaccharide with mixed α -1,6 and α -1,4 glucosidic linkages, followed isomaltooligosaccharides, and then maltose. The broad specificity observed has been observed in other oligo-1,6 glucosidases, however the high level of activity towards smaller isomaltooligosaccharides is particularly interesting as many enzymes of this classification tend towards hydrolyzing larger oligosaccharides (Yamamoto and Horikoshi 1990; Niu et al. 2017).

Another isomaltose hydrolyzing GH13_31 from *Bacillus subtilis* (now also called MalL, previously identified as *yvdL*, locus ID: BSU_34560) was characterized as part of larger study to determine the mechanisms of maltose metabolism (Schönert et al. 1998; Schönert et al. 1999). It was found that the *malL* gene was highly up-regulated during growth of *B. subtilis* on maltose, though catabolite repression was noted when cells were grown with both maltose and glucose. Furthermore, *malL* induction was observed during *B. subtilis* growth on more complex substrates possessing α -1,4 bonds, such as amylose, starch, and even glycogen (Schönert et al. 1999) Deletion of the *malL* gene resulted in an inability to utilize maltose (Schönert et al. 1998). Purified MalL was observed to have the greatest affinity for maltose, then isomaltose, and finally sucrose. The V_{MAX} ($\mu\text{mol min}^{-1} \text{mg protein}^{-1}$) for these substrates is reversed, with MalL cleaving sucrose fastest, then isomaltose and finally maltose. Almost no activity was observed on larger isomaltooligosaccharides (Schönert et al. 1999). In addition to these substrates, some activity was observed on *p*-nitrophenyl- α -D-pyranoside (PNPG) (Schönert et al. 1998).

Later studies involving *B. subtilis* MalL analyzed 268 single amino acid mutations and identified four that altered activity, Val200Ala, Val200Ser, Val200The, and Gly202Phe. The Val200Ser mutation led to an approximately two-fold reduction in K_M and a 1.2-fold increase in K_{CAT} while the Gly202Phe variation experienced a 2.2-fold reduction in K_M and a nearly five-fold reduction in K_{CAT} . Structural analysis indicated that the Val200Ser mutation induced additional hydrogen bond formation, thereby reducing conformational states and enzyme activity. Alternatively, the Gly202Phe mutation increased the flexibility of MalL, which resulted in increased energy requirements during the transition stage and a reduction in K_{CAT} .

Biochemical characterization of *Bifidobacterium breve* Ag11 and Ag12 reported high specific activity towards isomaltose and panose (Pokusaeva et al. 2009). No activity was observed on

maltose or other substrates with α -1,4 bonds present. Additionally, activity on isomaltotriose was significantly reduced for both Agl1 and Agl2. Interestingly, growth analysis of *B. breve* indicates that it can utilize both isomaltose and isomaltotriose as primary carbon sources, with culture grown on isomaltotriose exhibiting increased growth over that of isomaltose. Enzyme kinetics analyses revealed that both enzymes have similar temperature and pH optima, but the Agl1 protein broader ranges before experiencing a loss of activity. The authors report that Southern hybridization with the *agl* sequences indicated the presence of a homologous *agl* gene in other *Bifidobacterium* species, and a *Bifidobacterium adolescentis* genomic library screen identified the *aglA* and *aglB* genes as putative α -glucosidases (Van Den Broek et al. 2003). Later metabolic studies argued that members of *Bifidobacterium* preferentially hydrolyze larger oligosaccharides with high degrees of polymerization, which may provide a source of α -1,6 linkages for degradation by the *agl* gene families (Andersen et al. 2013; Hu et al. 2013).

One additional enzyme with high relative isomaltase activity was identified from *Bacillus cereus* (*mall*, locus ID: BAA11354.1) (Suzuki and Tomura 1986; Kizaki et al. 1993; Watanabe et al. 2001). While the enzyme was categorized as an oligo-1,6-glucosidase, it was most active on isomaltose with decreasing activity as the length of the oligosaccharide increased (Suzuki and Tomura 1986). Additionally, no notable activity was observed on α -1,4 glycosidic linkages Site-directed mutational analysis allowed for the identification of three catalytic residues; Asp199, Glu255 and Asp329, and two substrate binding residues; His103 and His328 (Watanabe et al. 2001). All of these residues are conserved amongst the characterized isomaltases summarized in this review, and provide the framework for optimization efforts if these or related enzymes were to be used industrially.

FUTURE OUTLOOK

The α -diglucosides are becoming increasingly prevalent in consumer goods as their beneficial properties are being discovered (Ogawa et al. 1998; Sanz et al. 2005; Gerschenson et al. 2017; Mooradian et al. 2017). This has led to an increase in research regarding α -diglucoside production. However, as they become more prevalent in food and consumer goods it will become increasingly important to understand how these sugars influence human health, particularly the gut microbiome. As has been previously observed with increased trehalose consumption, there may be unforeseen consequences regarding pathogenicity and virulence of gut bacteria (Dalwai et al. 2006; Collins et al. 2018). From an industrial standpoint, engineering organisms to generate these metabolites requires a complete understanding of their production, transport, and degradation (Keasling 2010; Abe et al. 2017). Therefore, a complete understanding of both biosynthesis and degradation are integral from an industrial standpoint. Below we propose four major focus questions to direct future work to identify and characterize mechanisms of bacterial α -diglucosides metabolism, with one goal being to accelerate the development of biomedicine and biotechnology applications.

What is the prevalence of bacterial α -diglucoside metabolic enzymes in diverse microbial consortia?

Currently, one substantial challenge that hinders identification of α -diglucoside metabolic enzymes is homology. As outlined in this review the amino acid sequence of trehalases, kojibiose phosphorylases, and isomaltases may only have moderate levels of identity. However, physiological and biochemical activities are not necessarily correlated with sequence identity. For example, *C. japonicus* Tre37B shares 44% identity with the characterized *E. coli* TreA but has no detectible trehalase activity (Garcia et al. 2020). Identifying potential targets becomes increasingly difficult if protein identity is inherently small, which is the case for the nigerose active enzymes discussed in this review (< 25% identity). Physiological experiments used to identify additional organisms capable of metabolizing these rare carbohydrates can be used as a basis to predict additional homologous genes. Additionally, those organisms described within this review can be used to identify potential transporters; a critical step for future metabolic engineering.

How are bacterial α -diglucoside metabolic enzymes regulated?

Physiological data are distinctly under-represented in the available literature regarding α -disaccharide metabolism, and this is likely a consequence the difficulty predicting complete metabolic pathways for diglucoside utilization. Furthermore, it would take significant work to generate the mutant strains required to characterize diglucoside metabolism from a variety of bacteria. However, physiological data are going to be critical for identifying potential enzymes and transport mechanisms. For example, the *B. subtilis* isomaltose metabolic system has been physiologically characterized and through those experiments it was discovered that a putative isomaltose active enzyme was essential for isomaltose metabolism (Mangas-Sánchez and Adlercreutz 2015). A second example comes from the *C. japonicus* trehalose metabolic system, where phenotypic analysis found that Tre37A is required for trehalose degradation but the predicted trehalase Tre37B was dispensable (Garcia et al. 2020). Alternatively, the presence of detectible enzyme activity does not correlate with physiological relevance. This was observed with the *E. coli* YgjK enzyme, which was capable of hydrolyzing nigerose, but *E. coli* is incapable of utilizing nigerose as a sole carbon source (Tonozuka et al. 2004). Finally, three novel *C. japonicus* strains capable of metabolizing kojibiose, nigerose, and isomaltose have recently been identified, however, predictive genomic analyses have not yet identified genes likely to act on said carbohydrates and phenotypic data will be essential to identify beneficial mutations (Garcia et al. 2019). Therefore, future experiments to generate physiological data will complement *in vitro* data and facilitate *in silico* predictions to discover and characterize bacterial diglucoside metabolic enzymes.

How are α -diglucosides transported by bacteria?

Current knowledge indicates that α -diglucosides are degraded and the resulting glucose moieties enter glycolysis (Kanehisa 2000). Amino acid sequence analysis of characterized kojibiose phosphorylases, nigerose phosphorylases, nigerose hydrolases, and isomaltases suggested that these enzymes are largely cytoplasmic, suggesting a transport requirement that may be substrate specific (Almagro Armenteros et al. 2019). Previous work to engineer

E. coli to metabolize isomaltose not only an isomaltase obtained from *B. subtilis*, but also an isomaltose specific phosphotransferase system (PTS) component from *K. pneumoniae* (Abe et al. 2017). In the absence of a PTS, the transportation systems for kojibiose and nigerose are poorly understood. Operon analysis of the *T. brockii* genome indicated that there are possible permeases surrounding the *kojP* gene which may be important for kojibiose transport into the cell (Chaen et al. 1999). Similarly, predicted permeases can be found near the *L. phytofermentans cphy1874* gene and the *E. coli ycjT* genes (Nihira et al. 2012; Mukherjee et al. 2018). In addition to permeases, TonB-dependent transporters may be involved in carbohydrate metabolism. Genome analysis of the *C. japonicus* genome, which does not have a PTS, found the presence of a TonB-dependent receptor upstream of the *tre37A* gene (Garcia et al. 2019). While deletion of this TonB-dependent receptor gene produced no notable physiological effect, it is possible that the entire complex would need to be deleted to elicit a phenotype. Future work will not only need to identify α -diglucoside transporters, but also determine their substrate specificity to maximize their utility in biomedical or biotechnology applications.

What are the principles to most efficiently optimize bacterial α -diglucoside metabolic enzymes?

Study of the α -diglucosides kojibiose, nigerose, and isomaltose are primarily limited by their availability. Many of those enzymes involved in biosynthesis are promiscuous and produce a wide variety of products, or are also capable of degradation (Table 2, Table 3). Several studies have focused on generating mutations in these enzymes for more efficient production of the desired α -diglucoside, and include reducing the variety of products formed, increasing substrate K_M , V_{MAX} , and K_{CAT} , and minimizing the enzyme's ability to hydrolyze the desired product.

Closing Remarks

Bacterial species capable of utilizing diverse nutrient sources gain a competitive advantage over those species with more restricted nutrient sources (Guignard et al. 2017). Therapeutics such as prebiotics and probiotics take advantage of this competition to alter the nutrient and bacterial composition, with the goal of improving human health (Holscher 2017; Tsai et al. 2019). Despite being understudied, α -diglucosides show great promise as a prebiotic, in addition to being an alternative sweetener, diagnostic tool, or therapeutic (Murosaki et al. 1999; Sanz et al. 2005; Luo et al. 2017; Al-Otaibi et al. 2018). To capitalize on their potential, future research will need to emphasize not only the discovery of α -diglucoside metabolic enzymes, but characterizing their physiological function in order to engineer them for maximal utility in biomedical and biotechnology applications.

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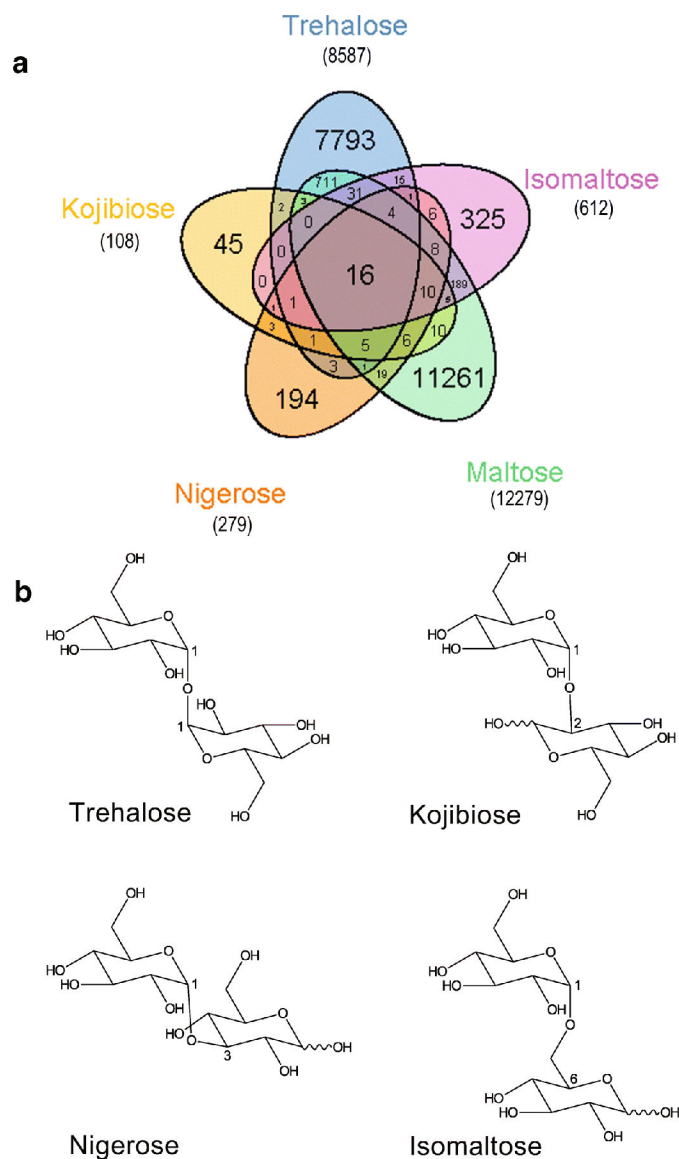
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KEY POINTS

1. α -diglucosides are increasingly important, but understudied bacterial metabolites.
2. Kinetically superior α -diglucosides enzymes require few amino acid substitutions.
3. *In vivo* studies are required to realize the biotechnology potential of α -diglucosides.



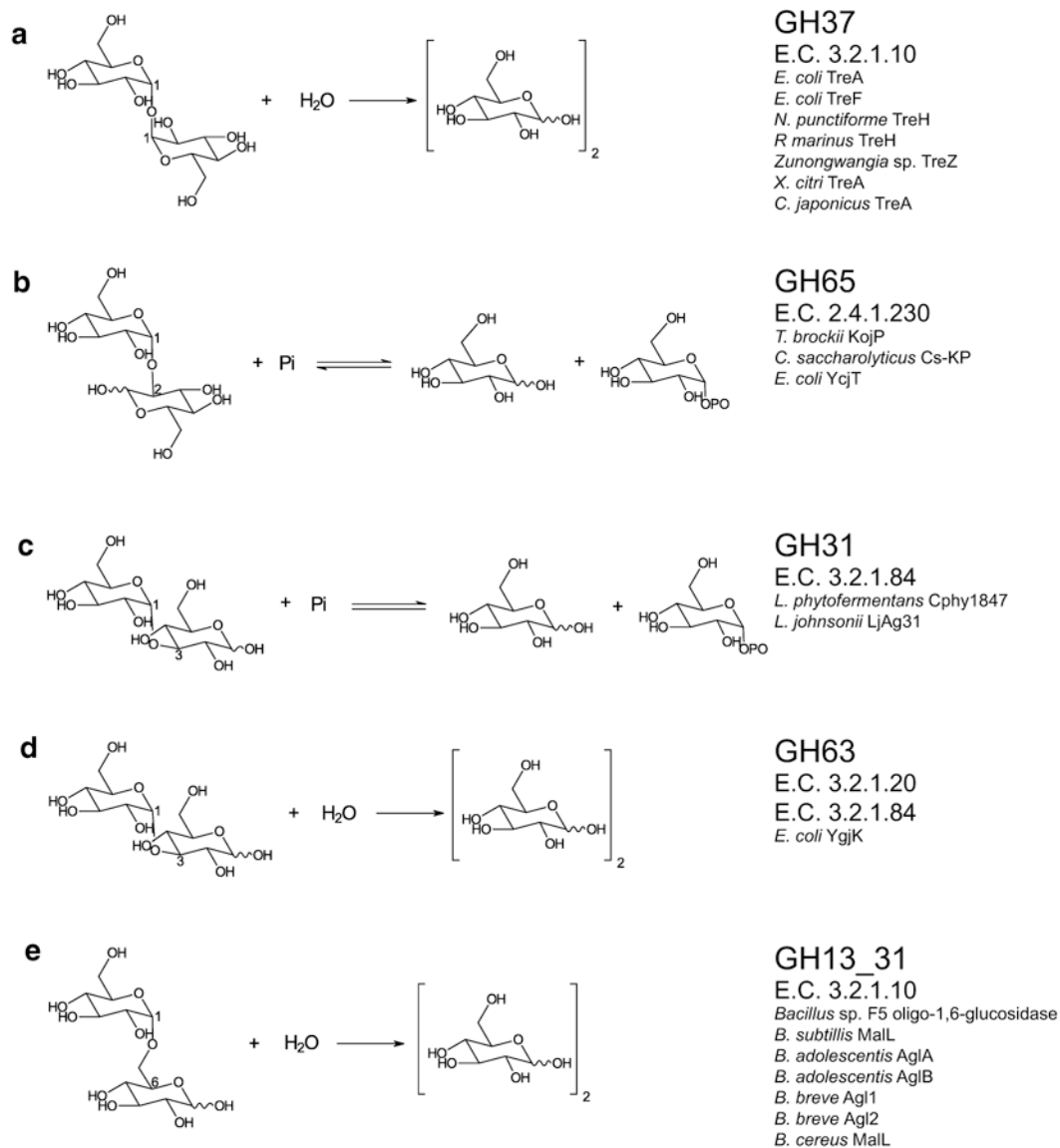


Fig. 2. Enzyme class reactions with representative members.

Enzyme reactions for those glycoside hydrolases (GH) and enzyme classes (E.C.) discussed.

Enzymes representative of their respective classes are listed for (a) GH37 trehalases, (b) GH65 kojibiose phosphorylases, (c) GH 31 nigerose phosphorylases, (d) GH63 nigerose hydrolases, and (e) GH 13_31 isomaltose hydrolases.

Table 1

Biochemical characterization of bacterial GH37 trehalases.

Enzyme	V _{MAX} ($\mu\text{M min}^{-1}$)	K _M (mM)	K _{CAT} (min^{-1})	Specific activity ($\mu\text{mol min}^{-1}$ mg^{-1})	pH	Temp ($^{\circ}\text{C}$)	Source
<i>E. coli</i> TreA	0.012	0.41	1.2×10^3	6.6×10^1	5.5	37	(Gibson et al. 2007)
<i>E. coli</i> TreF	54	1.9	N/A	6.0×10^1	6	N/A	(Horlacher et al. 1996)
<i>E. cloacae</i> TreA	200	1.47	6.3×10^3	1.4×10^3	5	55	(Adhav et al. 2019)
<i>N. punctiforme</i> TreH	N/A	18	N/A	2.5×10^{-2}	7.5	N/A	(Yoshida and Sakamoto 2009)
<i>R. marinus</i> TreH	81	0.16	N/A	1.3×10^1	6.5	88	(Jorge et al. 2007)
<i>Zunongwangia</i> sp. TreZ	N/A	0.99	1.6×10^4 5795	2.6×10^2	6	50	(Cheng et al. 2016)
<i>X. citri</i> sp. <i>citri</i> TreA	55.038	0.077	N/A	5.5×10^1	8	25	(Alexandrino et al. 2016)
<i>C. japonicus</i> Tre37A	N/A	N/A	N/A	7.3×10^3	6	20-30	(Garcia et al. 2020)

Table 2

Kojibiose phospholyase synthase (S) and phosphorolytic (P) activities.

Enzyme	V _{MAX} ($\mu\text{M min}^{-1}$)		K _M (mM)		K _{CAT} (min^{-1})		Specific activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)		pH		Temp ($^{\circ}\text{C}$)		Source
	S	P	S	P	S	P	S	P	S	P	S	P	
<i>T. Brockii</i> KojP	N/A	N/A	3.52	0.77	3.9×10^3	N/A	N/A	71.3	5.5	5.5	65	65	(Chaen et al. 1999)
<i>C. saccharolyticus</i> Cs-KP	N/A	N/A	N/A	N/A	N/A	N/A	N/A	7.34	5.5	6	75	85	(Yamamoto et al. 2011)
<i>E. coli</i> YcjT	N/A	N/A	1.7	1.05	48	66	N/A	N/A	N/A	N/A	N/A	N/A	(Mukherjee et al. 2018)

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Table 3

Biochemical characterization of nigerose synthase (S) and degradative (D) activities.

Enzyme	V_{MAX} ($\mu\text{M min}^{-1}$)		K_M (mM)		K_{CAT} (min^{-1})		Specific activity ($\mu\text{mol min}^{-1}$ mg^{-1})		pH		Temp ($^{\circ}\text{C}$)		Source
	S	D	S	D	S	D	S	D	S	D	S	D	
<i>L. phytofermentans</i> Cphy1874	N/A	N/A	3.3	1.7	6600	4.0×10^3	54	32	7	7	40	40	(Nihira et al. 2012)
<i>B. adolescentis</i> BaSP mutant	N/A	N/A	305	N/A	10.2	N/A	N/A	14.5	N/A	N/A	N/A	N/A	(Franceus et al. 2019)
<i>E. coli</i> YgjK	N/A	N/A	1.7	N/A	1.9×10^3	N/A	N/A	N/A	5	N/A	42	N/A	(Kurakata et al. 2008)
<i>L. johnsonii</i> LjAg31	N/A	N/A	1.79	N/A	2×10^3	N/A	N/A	N/A	6	N/A	35	N/A	(Kang et al. 2009)

Table 4

Biochemical characterization of bacterial GH13_31 oligo-1,6 glucosidases with high relative isomaltase activity.

Enzyme	V _{MAX} ($\mu\text{M min}^{-1}$)	K _M (mM)	KCAT (min^{-1})	Specific activity ($\mu\text{mol min}^{-1}\text{mg}^{-1}$)	pH	Temp ($^{\circ}\text{C}$)	Source(s)
<i>Bacillus</i> sp. F5 oligo-1,6-glucosidase	N/A	N/A	N/A	35 ^a	6 – 6.5	45	(Yamamoto and Horikoshi 1990)
<i>B. subtilis</i> Mall	162	0.455	N/A	2.02	7	42	(Schönert et al. 1998; Schönert et al. 1999)
<i>B. adolescentis</i> AglA	228	1.05	N/A	N/A	6.6 ^a	37 ^b	(Van Den Broek et al. 2003)
<i>B. adolescentis</i> AglB	113	0.47	N/A	N/A	6.8 ^a	47 ^b	(Van Den Broek et al. 2003)
<i>B. breve</i> Agl1	35.9	8.3	1848	14.1	5.5 ^b	37 ^c	(Pokusaeva et al. 2009)
<i>B. breve</i> Agl2	42.3	9	2178	20.4	5.5 ^b	30 ^c	(Pokusaeva et al. 2009)
<i>B. cereus</i> Mall	N/A	3.0	N/A	229 ^a	5.2	62	(Suzuki and Tomura 1986)

^aReported in U/mg

^bValues reported for *pnp*-glucanpyranoside, noted to be similar for isomaltose

^cTemperature and pH values reported for palatinose and used for determination of isomaltose