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MiniReview

Carbohydrate metabolism in *Zymomonas mobilis*: a catabolic highway with some scenic routes

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Abstract

Sucrose, glucose and fructose are degraded in the Gram-negative bacterium *Zymomonas mobilis* via an anaerobic version of the Entner-Doudoroff pathway, to an equimolar mixture of ethanol and carbon dioxide. Sucrose is split extracellularly into glucose and fructose (or levan). The two sugars are transported into the cell via facilitated diffusion (uniport). A periplasmic enzyme, glucose-fructose oxidoreductase, provides the novel compatible solute, sorbitol, to counteract detrimental osmotic stress. Carbon flux and its regulation, and branches into anabolic pathways are discussed together with recent approaches to broaden the substrate range of the bacterium.

Keywords: Entner-Doudoroff pathway; Glucose facilitator; Glucose-fructose oxidoreductase; Carbon flux; Ethanol; Metabolic engineering

1. Introduction

Zymomonas mobilis is an anaerobic, Gram-negative bacterium producing ethanol from glucose via the Entner-Doudoroff (2-keto-3-deoxy-6-phosphogluconate, KDPG) pathway in conjunction with the enzymes pyruvate decarboxylase and alcohol dehydrogenase (Fig. 1). Ethanol and carbon dioxide are the main products of catabolism when cells grow anaerobically on glucose. The microorganism was originally discovered in fermenting sugar-rich plant saps, e.g. in the traditional pulque drink (from agave sap) of Mexico, in palm wines of tropical Africa, or in ripening honey [1]. Interest in *Z. mobilis* has been revived in recent years and the organism is successfully used for fuel ethanol production. It shows higher ethanol productivity (3–5-fold) than yeasts with an ethanol yield from glucose of up to 97% of the theoretical maximum value [2]. Other favorable traits such as simple growth requirements, high sugar tolerance (up to 400 g/l) and resistance to high ethanol concentrations (up to 12%) would make the bacterial organism a serious competitor for the traditional yeasts if its narrow substrate range could be overcome [1–3]. Attempts to transform *Z. mobilis* into an efficient ethanol producer from abundant and renewable carbon sources (wood, straw, inedible plant parts, milk whey, etc.) are being carried out and have been reviewed earlier. For further in-depth treatises, the reader is referred to reviews on the microbiology [1], the taxonomic position [4], and the biotechnological use [3,5–8] of *Z. mobilis*. In spite of its powerful fermentative capabilities, *Z. mobilis* has retained functions of the electron transport chain and grows well in microaerobic environments [9], which

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underlines the taxonomic position of the organism with clearly aerobic close relatives (*Gluconobacter*, *Acetobacter*) [4].

Z. mobilis catabolizes only D-glucose, D-fructose and sucrose as sole carbon and energy sources [1]. Growth on sucrose is accompanied by the extracellular formation of the fructose oligomers levan and sorbitol with significant reduction in ethanol formation. Sorbitol is also formed and appears in the medium when the cells grow on mixtures of glucose and fructose exceeding 5% of each sugar (for review see [7]). Sugar metabolism of *Z. mobilis* appears like a 'metabolic highway' with few side routes branching off. This MiniReview deals with the different steps from outside the cell (cleavage of sucrose), carbohydrate uptake systems and carbon flux to ethanol, carbon dioxide and to by-products.

2. Extracellular and periplasmic enzymes of carbohydrate metabolism (sucrose, sorbitol, gluconate)

When *Z. mobilis* grows on sucrose, it converts the disaccharide into glucose and fructose using up to three sucrose-splitting enzymes: an extracellular levansucrase (LevU, forming the fructose oligosaccharide levan and glucose), an extracellular invertase (InvB), forming glucose and fructose [10–12], and a second invertase (InvA) whose exact role and location in *Z. mobilis* is unclear. The cloned *invA* gene is expressed as intracellular invertase activity in *E. coli* [13] but it remains unclear if the enzyme is of importance in *Z. mobilis* as sucrose is unlikely to reach the cell's interior. LevU and InvB (also known as SacB and SacC) share a high degree of identical amino acid residues, their genes are clustered together on the chromosome and it is likely that the two genes are of common origin [14–16]. Both proteins lack signal peptides typical for many other exported proteins. Instead, two genes were recently identified [17,18] that are thought to be involved in a specific protein export system, although the exact manner of export needs to be unravelled. Whether LevU and InvB are truly extracellular or are retained with the cell surface is still unclear.

Glucose-fructose oxidoreductase (GFOR), an NADP-containing enzyme, is unique to *Z. mobilis* [19]. The mature enzyme is located in the periplasm (Fig. 1) [20,21]. It converts glucose to gluconolactone and fructose to sorbitol. Gluconolactone is then converted by gluconolactonase, another periplasmic enzyme [22], to gluconic acid. Both enzymes are major constituents of the periplasm forming roughly 20–30% of the total protein in this compartment (T. Wiegert and G.A. Sprenger, unpublished results). Gluconic acid is taken up by the cells (the putative gluconate carrier of Fig. 1 has not been studied further) and can be completely degraded (as a co-substrate) to ethanol and acetic acid, although it is not a sole carbon source for *Z. mobilis* [23]. Possibly, *Z. mobilis* cannot form fructose-6-P and/or other gluconeogenic compounds from this carbon source (neither are gluconeogenic compounds such as glycerol, pyruvate or ethanol used as carbon sources) and therefore has no access to metabolites for cell wall formation. The physiological role of GFOR lies in the provision of sorbitol, a novel compatible solute for bacteria [24]. When the cells are stressed with high sugar concentrations (either physiological sugars or maltose, which is not taken up or metabo-

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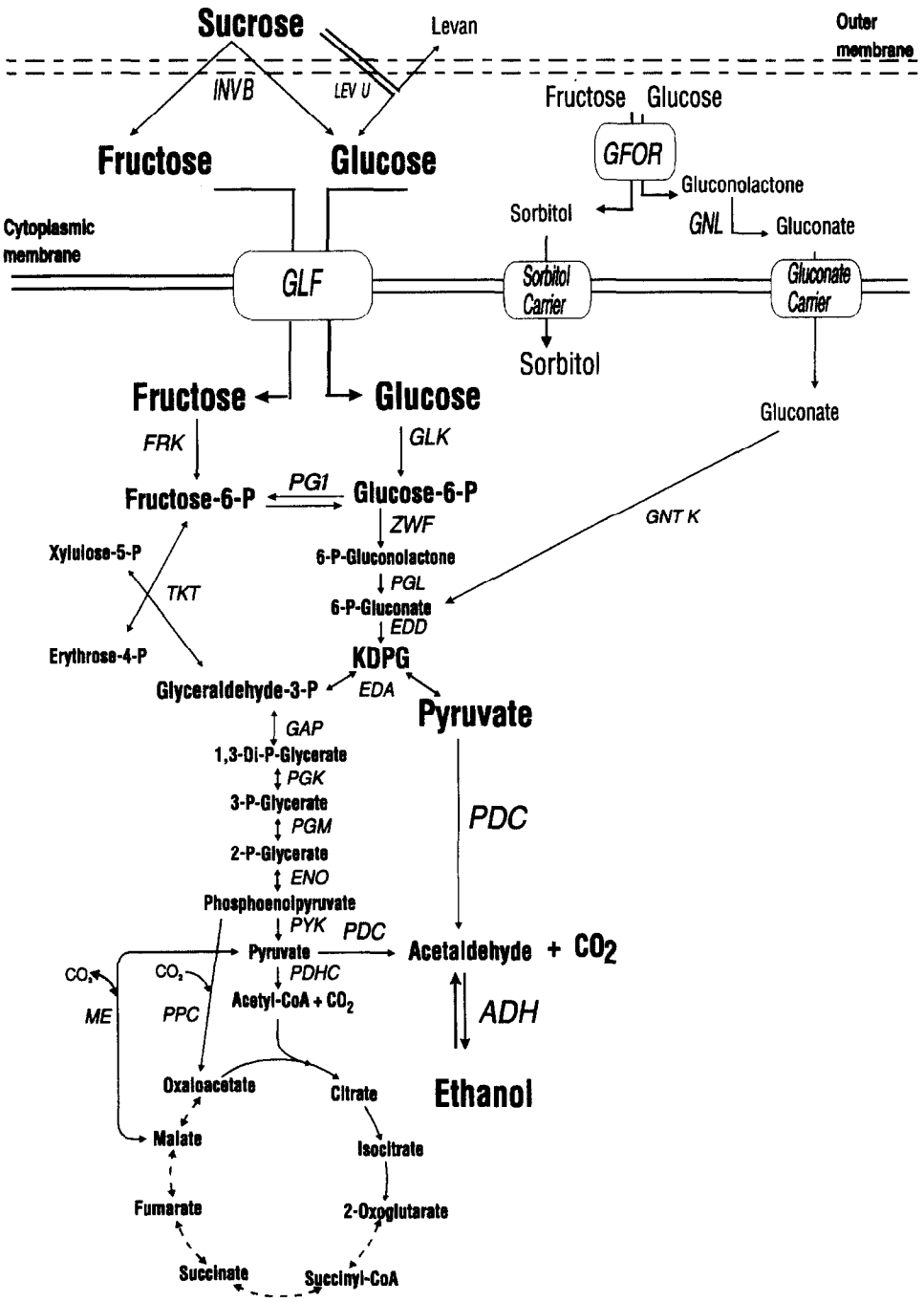
Fig. 1. Major carbohydrate pathways in *Zymomonas mobilis*. Sucrose is split extracellularly by the action of two enzymes (InvB and LevU) into glucose and fructose (or levan). The two hexoses can enter the cell via the common uniport system (GLF) or are converted by the periplasmic GFOR into sorbitol and gluconolactone. Sorbitol is transported into the cell by an accumulative system (sorbitol carrier [24]), gluconate uptake is not known in detail but a transporter has to be assumed (gluconate carrier). The enzymes of the KDPG pathway are written in three-letter code next to the arrows that symbolize the reaction steps. Two arrows indicate reactions that are, in principle, reversible. Dashed lines in the TCA cycle indicate missing reactions, the transketolase (TKT) reaction is the entrance to the pentose-phosphate pathway which is incomplete (discussed in the text). Abbreviations (in alphabetical order): ADH, alcohol dehydrogenases I and II; EDA, 2-keto-3-deoxy-gluconate aldolase; EDD, 6-phosphogluconate dehydratase; ENO, enolase; FRK, fructokinase; GAP, glyceraldehyde 3-phosphate dehydrogenase; GFOR, glucose-fructose oxidoreductase; GLF, glucose facilitator; GLK, glucokinase; GNL, gluconolactonase; GNT K, gluconate kinase; INV B, invertase B; KDPG, 2-keto-3-deoxy-6-phosphogluconate; LEV U, levansucrase; ME, malic enzyme; PDC, pyruvate decarboxylase; PDHC, pyruvate dehydrogenase complex; PGI, phosphoglucose isomerase; PGK, phosphoglycerate kinase; PGL, phosphogluconolactonase; PGM, phosphoglyceromutase; PPC, phospho*enol*pyruvate carboxylase; PYK, pyruvate kinase; TKT, transketolase; ZWF, glucose 6-phosphate dehydrogenase (zwischenferment).

lized), they accumulate sorbitol intracellularly up to 1 M [24] to counteract the detrimental effects of dehydration exerted by high external osmolarity. A mutant strain, isolated as not producing sorbitol from sucrose [25], was recently shown to be GFOR-deficient and unable to grow in the presence



of 1 M sucrose unless sorbitol was added [26]. Sorbitol, however, does not protect the cells from salt stress, as they remain unable to grow in media with NaCl above 2% [1,24]. GFOR has an unusually low affinity for fructose (K_m above 400 mM [19]). In nature, such high fructose concentrations are rare but may occur in sugar-rich plant saps which frequently fall dry, or in honey – both well-known habitats for *Z. mobilis* [1]. Desiccation of plant saps should lead to an increase in sucrose concentration and thence of its moieties glucose and fructose, by the action of invertase. Thus, an increasing concentration of fructose would be a sign for future osmotic stress for *Z. mobilis*. If GFOR has both its substrates present to form sorbitol efficiently, the polyol can be accumulated in the cells to counteract the effects of lowered water activity.

Why is GFOR located in the periplasm? Most likely, this compartment offers the unique possibility to have both glucose and fructose present in saturating amounts. High fructose concentrations are unlikely to occur in the interior of the cell concomitant with the presence of high glucose because of the discrimination of fructose by the sugar uptake system (glucose facilitator, GLF, Fig. 1, see also below), which clearly prefers glucose over fructose [27,28]. The periplasmic location of GFOR, however, challenges the cell with the task of exporting the valuable cofactor NADP to the periplasm. GFOR is formed as a preprotein with an unusually long signal peptide of 52 amino acid residues [29]. This signal peptide, however, is not essential for GFOR export as it can be exchanged for other signal peptides [26]. The mode of NADP export in *Z. mobilis* is not yet understood (as for other microorganisms), but it is unlikely to occur bound to preGFOR while it is translocated to the periplasm [26].

3. Carbohydrate uptake systems

Z. mobilis is unique among prokaryotes in that it relies solely on a facilitated diffusion system (uniport) to transport glucose and fructose without expending metabolic energy [27]. The gene for a glucose facilitator (*glf*) was identified by its great similarity to eukaryotic facilitators and bacterial symport systems [30]. When expressed in suitable *E. coli* mutants, *glf* conferred glucose uptake on its host [31]. Proof that *glf* encodes a true facilitator (GLF) was provided soon afterwards [28,32]. Besides glucose, fructose is also transported [28], and recently mannose and xylose have been added to the substrate list of GLF, although these two sugars do not serve as carbon sources for wild-type cells (P. Weißer, R. Krämer and G.A. Sprenger, Appl. Environ. Microbiol., submitted for publication). GLF has the highest affinity for glucose (K_m 2–4 mM) ([28,32]; Weißer et al., submitted) and, in descending order, mannose (8 mM), xylose and fructose (40 mM each; [28]; Weißer et al., submitted). Transport activities are about equal for glucose, mannose and fructose, whereas xylose is transported nearly twice as fast (Weißer et al., submitted).

Sucrose, however, is unlikely to be a substrate for GLF ([32]; Weißer et al., submitted), and whether sucrose is taken up into the cell at all is still questionable since levansucrase and invertase should prevent the sugar from reaching the cytoplasmic membrane.

Sorbitol (see Section 2) is accumulated via an energy-dependent system (sorbitol carrier; Fig. 1) [24]. It is not known how gluconic acid is taken up [23].

4. Entner-Doudoroff and pentose-phosphate pathways, anaplerotic reactions

Z. mobilis uses the Entner-Doudoroff (KDPG) pathway for catabolism of its carbon sources [33]. The pathway is so efficient that up to 1 μmol of glucose is converted to ethanol and carbon dioxide per min per mg of cell protein (even in cell-free extracts), with the production of only one net mol of ATP per mol of glucose consumed in whole cells [34,35]. All KDPG proteins have been purified [36], making *Z. mobilis* one of the few bacteria where this has been achieved, and most of the respective genes have been identified and their DNA sequences established [8]. The paramount activities of the KDPG pathway leave the cell with problems to channel metabolites into its anabolic pathways (only about 2% of the carbon source ends up in biomass [2]). The pentose-phosphate pathway is incomplete as no transaldolase activity can be detected [37,38]; however, the cell manages to provide building blocks for

nucleic acids, vitamins, amino acids and cell wall biosynthesis by a combination of the (weak) transketolase, ribose-5-P isomerase and ribulose-5-P epimerase acting in subsequent steps on fructose-6-P and glyceraldehyde-3-P (Fig. 1) [37]. The strong KDPG activities made it difficult to detect anabolic and anaplerotic reactions that use the building blocks PEP and pyruvate. Recently, a pyruvate dehydrogenase complex was detected which is unique as it functions under strictly anaerobic conditions and has to compete with the overwhelming pyruvate decarboxylase activity while showing similar affinity to pyruvate [39]. A complete TCA cycle is absent; the cell uses several enzymes to convert citrate to oxoglutarate, additional anaplerotic reactions are carried out by a phosphoenolpyruvate carboxylase and a malic enzyme (Fig. 1) [40].

With a few exceptions (see below), all KDPG enzyme activities appear to be present constitutively and their activities are not considered to be regulated by feed-back inhibition (allosteric control) or by other modes of regulation [34,41,42]. Growth on fructose augments the activity of fructokinase about twofold [43] and also doubles phosphoglucose isomerase activity [44], likewise the specific mRNA transcripts of the respective genes and of *glf*-specific mRNA were augmented when cells were grown on fructose. Together, this might ensure that sufficient transport protein and enzyme activities are present to cope with the somewhat handicapped fructose metabolism [45].

5. Carbon flux and flux control

The unique features of *Z. mobilis* sugar metabolism have attracted considerable interest as the organism can serve as an excellent model system for investigations of glycolytic flux [35,41,42,46] and its regulation (more than 95% of glucose is converted into an equimolar mixture of ethanol and carbon dioxide). The enzymes involved in the KDPG pathway constitute up to 50% of the cells' total protein [36] and each glycolytic gene is highly expressed [34]. As regulation of transcription and control of enzyme activity appear to be of minor importance, it became interesting to find out how the cell adjusts its KDPG machinery to ensure optimal functionality. Differential stability of polycistronic mRNAs seems to play a pivotal role in ensuring stoichiometric relations of the different enzymes in the cell (see [47] and references therein). As the genes for the KDPG pathway enzymes and sugar uptake (*glf*) are now available, they can be expressed and/or combined in various ways to study carbon flux through the KDPG pathway, measured by the rate of CO_2 evolution [35] or by NMR [41,42]. Four possible checkpoints were identified: the carbohydrate uptake system (GLF), the sugar kinases (glucokinase and fructokinase), phosphoglucose isomerase for growth on fructose, and the glucose-6-P dehydrogenase. Recently [35] it was reported that the glycolytic flux is mainly controlled by the glucose-6-P dehydrogenase activity ($C^J_{\text{G-6-P-dehydrogenase}} = 0.4$). However, GLF and glucokinase also contribute to the flux control [41,42].

6. New pathways through metabolic engineering

Approaches to broaden the narrow substrate and product range of *Z. mobilis* were made soon after the organism became popular through the pioneering work of Rogers and coworkers [2]. Although many attempts met with little success in the past (for references see [3,8]), two groups reported recently on the engineering of pathways for xylose [37,38] and for mannose catabolism (Weißer et al., submitted), both sugars being constituents of plant hemicelluloses. Growth on pentose sugars is prohibited (besides the lack of specific peripheral enzymes) by the lack of transaldolase in the pentose-phosphate pathway [37]. The introduction of heterologous genes for transketolase in conjunction with the genes for xylose isomerase and xylulokinase allowed a partial conversion of xylose to ethanol and carbon dioxide [37]. The prediction then was that an additional transaldolase should allow growth on xylose [37]; following this path, a xylose catabolic pathway was established recently (μ about 0.06 h^{-1}) [38]. Growth on mannose was accomplished by solely introducing the phosphomannose isomerase (*pmi*) gene of *E. coli* (μ about 0.07 h^{-1}). Uptake of mannose is via GLF (see above), and phosphorylation by a side-reaction of fructokinase. Augmenting fructokinase activity resulted in acceleration of growth in *pmi*-positive strains (μ up to 0.25 h^{-1}) (Weißer et al., submitted).

7. Conclusions

Powerful techniques of protein chemistry, molecular biology (cloning and expression of homologous and heterologous genes), ^{13}C- [48] and ^{31}P-NMR methodologies (carbon flux measurements), advanced fermentation engineering, and modelling of glycolytic flux in *Z. mobilis* have already delivered a plethora of data on the biochemistry and genetics of carbohydrate metabolism in this bacterium. The combination of the different approaches will certainly improve understanding and engineering of the main metabolic highway but this should not distract too much attention from some scenic and unique routes (GFOR, sugar uptake, hopanoid biosynthesis) present in this organism.

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