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Revealing *in vivo* glucose utilization of *Gluconobacter oxydans* 621H Δ*mgdh* strain by mutagenesis

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ABSTRACT

Gluconobacter oxydans, belonging to acetic acid bacteria, is widely used in industrial biotechnology. In our previous study, one of the main glucose metabolic pathways in *G. oxydans* 621H was blocked by the disruption of the *mgdh* gene, which is responsible for glucose oxidation to gluconate on cell membrane. The resulting 621H Δ*mgdh* mutant strain showed an enhanced growth and biomass yield on glucose. In order to further understand the intracellular utilization of glucose by 621H Δ*mgdh*, the functions of four fundamental genes, namely glucokinase-encoding *glk1* gene, soluble glucose dehydrogenase-encoding *sgdh* gene, galactose–proton symporter-encoding *galp1* and *galp2* genes, were investigated. The obtained metabolic characteristics of 621H Δ*mgdh* Δ*glk1* and 621H Δ*mgdh* Δ*sgdh* double-gene knockout mutants showed that, *in vivo*, glucose is preferentially phosphorylated to glucose-6-phosphate by glucokinase rather than being oxidized to gluconate by soluble glucose dehydrogenase. In addition, although the galactose–proton symporter-encoding genes were proved to be glucose transporter genes in other organisms, both *galp* genes (*galp 1* and *galp2*) in *G. oxydans* were not found to be involved in glucose uptake system, implying that other unknown transporters might be responsible for transporting glucose into the cells.

1. Introduction

Acetic acid bacteria are a large group of Gram-negative, aerobic, and rod-shaped acidophilic organisms that are generally characterized by their ability to incompletely oxidize a wide range of alcohols and sugars. Many of these bacteria are originally isolated from the plant environments such as fruits and flowers as well as from alcoholic beverages and soft drinks. Among them, *Gluconobacter oxydans* is one of the well-studied bacteria and is generally used to develop industrial fermentation processes for the production of vitamin C, miglitol, dihydroxyacetone (DHA) and several other compounds (Adachi et al. 2003; Asai 1968; Gupta et al. 2001; Yang et al. 2008).

Notwithstanding glucose is the favorable carbon source for a large number of obligated processes, its utilization by *G. oxydans*, which prefers glycerol, sorbitol or mannitol, remains challenging. During growth on glucose medium, glucose is generally utilized through two different ways. The first one includes glucose uptake, its intracellular oxidation and its further dissimilation *via* metabolic pathways such as the pentose phosphate pathway and the Entner–Doudoroff pathway (Pronk et al. 1989); The other way is the direct oxidation of glucose by membrane-bound glucose dehydrogenase (mGDH) into gluconate which can be further oxidized to ketogluconate (Holscher et al. 2009; Matsushita et al. 1989). The latter has been identified as the major way of glucose utilization by *G. oxydans* during typical fermentation processes. The extremely low pH value caused by gluconate and ketogluconate are usually harmful to the cell growth and the catalytic activity of resting cells of *G. oxydans* (Silberbach et al. 2003; Zhu et al. 2011). In industrial production, it is preferable to obtain large amount of *G. oxydans* cells as biocatalyst on relatively inexpensive carbon sources. Previous studies indicated that the mutant strain deficient in mGDH could exhibit enhanced growth rate and biomass yield on glucose (Gupta et al. 1997; Krajewski et al. 2010; Lu et al. 2012). In addition, the absence of mGDH eliminated the extracellular glucose oxidation and thus could facilitate the study of intracellular utilization of glucose. Activities of enzymes involved in the central metabolic pathways were usually analyzed *in vitro* in routine study of glucose metabolism in *G. oxydans*, which, however, do not necessarily reflect the *in vivo* status of glucose metabolism (Attwood et al. 1991; Kitos et al. 1958; Rauch et al. 2010). Owing to the absence of some key glycolytic and Krebs cycle enzymes (Prust et al. 2005), glucose is predominantly catabolized through the pentose phosphate pathway in *G. oxydans*. There are two propositional distinct intracellular pathways in *G. oxydans* before glucose is being degraded into the intermediate metabolite 6-phosphogluconate (6PG). Indeed,

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after its transportation into the cell, glucose can either be oxidized by soluble glucose dehydrogenase (sGDH) into gluconate and then be phosphorylated to 6PG, or be phosphorylated by hexokinase into glucose-6-phosphate and then be dehydrogenated to 6PG. Subsequently, each of the two resulting transitional metabolites is incorporated through the pentose phosphate pathway. But, which pathway is dominantly used by mGDH-disrupted *G. oxydans* mutant still remains unknown.

Generally, in Gram-negative bacteria such as *Escherichia coli*, the uptake and phosphorylation of glucose are carried out mainly by the phosphotransferase system (PTS), which catalyzes the concomitant uptake and phosphorylation of its substrates. Besides, glucose can also be taken up by the constitutively expressed galactose transporters and then phosphorylated by glucokinase. The complete genome of *G. oxydans* DSM 2343 (ATCC 621H) reveals that some components of PTS are encoded, but not sugar-specific proteins (EIIB and EIIC) (Prust et al. 2005). It may infer that the utilization of glucose by *G. oxydans* might be proceeded by a non-PTS mechanism. It has been reported that in a PTS-inactive mutant strain such as *E. coli*, galactose–proton symporter (Galp) has replaced the transport function of the $IICB^{Glc}$ PTS protein (Flores et al. 2002; Hernandez-Montalvo et al. 2003; Wang et al. 2006). The genome annotation revealed that there are two chromosomal galactose–proton symporter in *G. oxydans* 621H, encoded by GOX0808 (*galp1*) and GOX1971 (*galp2*), respectively. The functions of the two genes, however, were not experimentally identified.

Although the glucose metabolism pathway is well characterized in *G. oxydans* previously, much less is known about the utilization of glucose as main carbon source *in vivo* of *G. oxydans* 621H Δ*mgdh*, which could eliminate the *in vitro* glucose oxidation (one of the main glucose metabolism pathway in *G. oxydans*). In this study, to understand the glucose utilization of *G. oxydans* 621H Δ*mgdh*, the functional characterizations of the four key enzymes involved in glucose metabolism were studied. To this purpose, glucokinase-encoding gene GOX2419 (*glk1*), soluble glucose dehydrogenase-encoding gene GOX2015 (*sgdh*), galactose–proton symporter-encoding genes GOX0808 (*galp1*) and GOX1971 (*galp2*) of *G. oxydans* 621H Δ*mgdh* strain were knocked out respectively. The physiological and metabolic properties of the resulting double-gene knockout mutants grown on glucose medium were therefore investigated to better comprehend the intracellular metabolism of glucose by *G. oxydans*.

2. Materials and methods

2.1. Strains and growth conditions

The list of strains and plasmids used in this study is given in Table 1. *Gluconobacter* strains were grown at 30 °C, 220 rpm in yeast–sorbitol (Y–S) medium (yeast extract 20 g/L, sorbitol 80 g/L and other factors, pH 6.0), yeast–glucose (Y–G) medium (yeast extract 20 g/L, glucose 20 g/L and other factors, pH 6.0) or yeast–galactose (Y–Ga) medium (yeast extract 20 g/L, galactose 20 g/L and other factors, pH 6.0). *E. coli* strains were cultivated at 37 °C in Luria-Bertani medium (yeast extract 5 g/L, peptone 10 g/L, NaCl 10 g/L) containing appropriate antibiotics.

2.2. General genetic manipulations

Primers used in this study were listed in Table 2. DNA manipulation was performed according to standard protocols (Sambrook and Russell 2001). Genomic DNAs of *Gluconobacter* strains were isolated from cells grown to the mid-exponential phase in Y–S medium with a TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China). For PCRs, genomic DNA isolated from *Gluconobacter* strains was used as a template. *LA* Taq polymerase (TaKaRa Biotechnol., Dalian, China) was used for PCR amplification in test reactions and reverse transcription PCR (RT-PCR).

2.3. Double-gene disruption in G. oxydans 621H and phenotype analysis

The mutant strains with double-gene disruption in *G. oxydans* 621H were constructed. Respective deletions of *glk1* (GOX2419), *sgdh* (GOX2015), *galp1* (GOX0808), *galp2* (GOX1971) genes from the chromosome of *G. oxydans* 621H Δ*mgdh* (GDHK) was performed using the strategy developed by Zhu et al. with minor modifications (Zhu et al. 2011). For the deletion of the *glk1* gene, two fragments for the homologous recombination and the kanamycin resistance gene were obtained by PCR using the primers shown in Table 2. The PCR products were then digested and ligated to a suicide plasmid pSUP202, yielding the gene replacement vector pSUP202 *glk1*::Km. The gene replacement was transferred into *G. oxydans* by triparental mating, using *E. coli* JM109 bearing the respective vector as the donor and *E. coli* HB101 bearing plasmid pRK2013 as the helper strain. The kanamycin/gentamycin-resistant colonies were isolated, and the gene disruption strain was confirmed by PCR. Deletion of the *sgdh*, *galp1*, *galp2* gene from 621H Δ*mgdh* was respectively performed in the same manner as described for the *glk1* gene deletion. For phenotypic assay, *G. oxydans* 621H Δ*mgdh* and double-gene deletion mutant strains were cultivated on Y–G or Y–Ga medium. Cell growth was monitored spectrophotometrically at 600 nm. Dry cell weight (DCW) was determined by collecting cell pellets from 100 to 200 mL of culture aliquots, washing with distilled water, and drying at 85 °C until constant weight. Glucose concentration was determined enzymatically with microplate spectrofluorometer (PowerWave XS/XS2, BioTek, Winooski, VT).

2.4. Enzyme assays

Gluconobacter cell extracts were prepared from fresh cultures in Y–G medium described above. The cells were washed, resuspended in disruption buffer (reaction buffer for enzyme assay including one tablet of protease inhibitor (EDTA-free)), and disrupted by ultrasonification in an ice bath. Cell debris were removed by centrifugation at 12,000 rpm for 30 min at 4 °C. Glucokinase activity was measured based on the formation of NADH in a reaction mixture containing cell extract, 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM glucose, 0.2 mM NAD^+, 2 mM ATP, 1.5 U glucose 6-phosphate dehydrogenase. Soluble glucose dehydrogenase activity was also measured based on the formation of NADPH. The reaction mixture contained cell extract, 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 0.2% Triton X-100 (in order to prevent NADPH oxidase activity) (Boutin 1986), 60 mM glucose, 0.5 mM $NADP^+$. Formation of NAD(P)H was monitored as the increase in absorption at 340 nm and a molar extinction coefficient of NAD(P)/H at 340 nm of 6.22 mM^{-1} cm^{-1} was used to calculate enzyme activity. Total proteins concentration was determined by Bradford method with bovine serum albumin as standard (Bradford 1976).

2.5. Detection of gene expression by quantitative real-time PCR (qRT-PCR)

For quantitative real-time PCR experiments, *G. oxydans* strains were grown to the late exponential phase in glucose medium. Total RNA was isolated using Trizol (Molecular Research Center, Cincinnati, OH) by following manufacturer's procedure. To remove residual DNA, total RNA was treated with DNase I for 30 min at 37 °C. RNA samples were reverse-transcribed with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) according to the manufacturer's instructions. Subsequently, the