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Phosphoenolpyruvate-dependent phosphorylation of sucrose by *Clostridium tyrobutyricum* ZJU 8235: Evidence for the phosphotransferase transport system

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ABSTRACT

The uptake and metabolism of sucrose, the major sugar in industrial cane molasses, by *Clostridium tyrobutyricum* ZJU 8235 was investigated and this study provided the first definitive evidence for phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) activity in butyric acid-producing bacteria. Glucose was utilized preferentially to sucrose when both substrates were present in the medium. The PEP-dependent sucrose: PTS was induced by growing *C. tyrobutyricum* on sucrose (but not glucose) as the sole carbon source. Extract fractionation and PTS reconstitution experiments revealed that both soluble and membrane components were required for bioactivity. Sucrose-6-phosphate hydrolase and fructokinase activities were also detected in sucrose-grown cultures. Based on these findings, a pathway of sucrose metabolism in this organism was proposed that includes the forming of sucrose-6-phosphate via the PTS and its further degradation into glucose-6-phosphate and fructose-6-phosphate.

1. Introduction

Production of butyric acid by saccharolytic clostridia species has been known for a long time (Péaud-Lenoël, 1952; Playne, 1985), but recent studies indicate a renewed interest in this fermentation (Zigová and Šturdik, 2000; Papoutsakis, 1984; He et al., 2005; Ramey and Yang, 2004). In particular, *Clostridium tyrobutyricum* has received much attention on account of the fact that it not only can be genetically manipulated with comparative ease (Zhu et al., 2005; Liu et al., 2006), but that is also has an ability to metabolize a wide range of carbohydrates. This capacity offers the potential for the biotechnological instead of oil-based production of butyric acid (Zhu et al., 2002; Huang et al., 2002; Liu and Yang, 2006; Jiang et al., 2009b). However, limited yields caused by high concentration of substrates and problems associated with organic acids toxicity have restricted process development. A greater understanding of the physiology and genetics of the acid-forming clostridia at the species level is now required. It will be helpful to develop the strains with improved properties, and optimize the growth conditions for the maximum product formation. Among these, a potentially important control point which has largely been ignored is the accumulation of fermentable substrates by the cells. In a sense, crucial to the future large-scale application of butyric acid fermentation in industry will be an improvement in the efficiency of converting a cheap substrate into the end product, where the first stage is the uptake of metabolizable carbon.

In general, the carbon source is usually used in relatively higher concentration compared to other medium components and thus has a high share in the raw material cost. Therefore, the exploitation of cheap, renewable carbon sources has been strongly preferred. Cane molasses, a byproduct of the sugar industry, contains approximately 35–50% (w/w) sucrose, which is much higher than the concentration of monosaccharides such as glucose and fructose (Zhu et al., 2008), and has been proven to be efficiently utilized by *C. tyrobutyricum* without hydrolysis (Jiang et al., 2009b). However, growth and metabolism of butyric acid bacteria are strongly inhibited when adding sucrose concentration up to 100 g l^{-1}, together with the increase of residualsugar (Jiang, unpublished data). It seems that the variety of sucrose concentrations would affect its utilization in turn. The effects of high sucrose concentration may be due to the influence of the osmotic pressure of the external culture medium, thus leading to the change in the growth of bacteria by determining sucrose uptake (Honecker et al., 1989). Unfortunately, little is currently known about the metabolism and regulation of sucrose in acid-forming clostridia. One possible metabolic path could involve the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), composed of two general cytosolic proteins, called Enzyme I and His-Protein (HPr), as well as a substrate-specific enzyme complex called Enzyme II, which is particularly widespread amongst anaer-

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obic bacteria (Postma et al., 1993). In this system, Enzyme I catalyzes the transfer of the phosphate group from phosphoenolpyruvate to a nitrogen atom of a histidine moiety in a small protein (HPr) according to reaction (1); in subsequent processes of the type represented in reaction (2), the appropriate Enzyme II catalyzes the transfer of phosphate from the phosphorylated protein (phospho-HPr) to its specific sugar acceptor:

$$\text{Phosphoenolpyruvate} + \text{HPr} \xrightarrow{\text{Enzyme I}} \text{phospho} - \text{HPr} + \text{pyruvate}, \quad (1)$$

$$\text{Phospho} - \text{HPr} + \text{carbohydrate} \xrightarrow{\text{Enzyme II}} \text{carbohydrate} - \text{phosphate} + \text{HPr}, \quad (2)$$

$$\text{Phosphoenolpyruvate} + \text{carbohydrate} \rightarrow \text{carbohydrate} - \text{phosphate} + \text{pyruvate}. \quad (3)$$

By this method the energy stored in the high energy phosphate bond in PEP is harnessed to drive the translocation of the substrate, with its concomitant phosphorylation, thus trapping the product within the cell. This PEP-dependent sucrose: PTS has been demonstrated in several other saccharolytic clostridia (Tangney et al., 1998; Nolling et al., 2001; Shimizu et al., 2002; Sharon and Valerie, 2005), where it may not only be involved in transport but also in metabolic regulation. Furthermore, the product of sucrose transport via the PTS will most likely be sucrose-6-phosphate, which should be further modified before it can enter the central metabolism. It was reported that the first enzyme, sucrose-6-phosphate hydrolase (S6PH), cleaves sucrose-6-phosphate to glucose-6-phosphate and fructose, then the second, an ATP-dependent fructokinase (FK), catalyzes the phosphorylation of the ketohexose to fructose 6-phosphate (Sharon and Valerie, 2005).

In the present work, we focused on the investigation of the existence of a PTS for uptake of sucorse and characterized sucrose transport and metabolism in *C. tyrobutyricum* ZJU 8235. These findings will be helpful for futher understanding of the control mechanism of carbohydrate assimilation in this organism.

2. Methods

2.1. Organism and culture conditions

Clostridium tyrobutyricum ZJU 8235 was stored in reinforced clostridial medium (Huang and Yang, 1998) and working cultures were grown in a clostridial basal medium, which contained (per liter of distilled water): 5 g yeast extract, 5 g peptone, 3 g $(NH_4)_2SO_4$, 1.5 g K_2HPO_4, 0.6 g $MgSO_4{\cdot}7H_2O$, 0.03 g $FeSO_4\,7H_2O$. Carbon sources were prepared as a separate anaerobic solution (20%, w/v) and added (6 g l^{-1}) to the basal medium after autoclaving. Incubations were performed at 37 °C in serum bottles. Batch fermentations were performed in a 5 l stirred-tank formentor containing 2 l of a medium with either sucrose or both sucrose and glucose as the substrate. Anaerobiosis was reached by initailly sparging the medium with nitrogen. A detailed description of the reactor operations has been given elsewhere (Jiang et al., 2009a).

2.2. Preparation of toluene-treated cells

Cells in logarithmic growth (1.1–1.5 mg dry weight of cells/ml) were harvested (40 ml) by centrifugation (12,000*g*, 10 min, 4 °C) and washed once with 100 mM sodium–potassium phosphate buffer (pH 7.2) plus 5 mM $MgCl_2$. Cells were suspended in 10 ml of buffer and stored on ice. One milliliter of cell suspension was treated with 30 μl of a toluene–ethanol mixture (1:9, v/v) as previously described (Kornberg and Reeves, 1972; Martin and Russell, 1986).

2.3. Preparation of cell-free extracts

Cells from 400 ml of culture harvested as described in Section 2.2 were sonicated for 30 min (SCIENIZ JY 92- II; microtip; 30% duty cycle; 0 °C), unbroken cell debris was eliminated by centrifugation (37,000*g*, 20 min, 4 °C), and cell-free extracts were stored at 4 °C before assays. Fractionation of extracts into soluble and membrane components was as described previously by Mitchell and Booth (1984).

2.4. Standard phosphotransferase assay

PEP-dependent phosphorylation of sucrose was determined by a coupled assay with lactate dehydrogenase (LDH, EC.1.1.1.27; Sigma). The assay mixture (1.0 ml) contained 100 mM sodium–potassium phosphate buffer (pH 7.2), 5 mM $MgCl_2$, 5 mM of PEP or 5 mM of ATP, 1 mM dithiothreitol (DTT), 0.2 mM of β-NADH (Sigma), 0.3 μg (2 units) of LDH, 30–150 μg dry weight of toluene-treated cells. The reaction was initiated by the addition of 5 mM carbohydrate, and the decrease in A_{340} was monitored at 37 °C in a recording spectrophotometer equipped with a thermostatically controlled cell compartment and automatic sample changer. A similar cuvette to which PEP (or ATP) was not added served as the control. In addition to achieving thermal equilibrium, this incubation period (which lasted from 5 to 20 min) ensured that any endogenous carbohydrate, which might be present in the cells, was consumed. The molar extinction coefficient of NADH was taken to be 6.22×10^3 l mol^{-1} cm^{-1} (Kornberg and Reeves, 1972).

2.5. Enzyme assays in cell-free extracts

The hydrolysis of sucrose to glucose and fructose was determined by an enzyme assay (St. Martin and Wittenberger, 1979). Sucrose-6-phosphate hydrolase activity was detected using the coupled enzyme assay, wherein NADPH, generated during the oxidation of glucose-6-phosphate formed in the assay, was measured. Fructokinase activity was determined as ATP-dependent phosphorylation of fructose. The 1 ml reaction mixture contained 100 mM sodium–potassium phosphate buffer (pH 7.2), 5 mM $MgCl_2$, 5 mM ATP, 0.2 mM $NADP^+$, 6.4 U of glucose-6-phosphate dehydrogenase (EC 1.1.1.49; Sigma), 2 U of hexokinase (EC 2.7.1.1; Sigma), 2 U of phosphoglucose isomerase (EC 5.3.1.9; Sigma), 4 mM sucrose, and 100 μl of cell-free extracts.

3. Results

3.1. Sugar utilization by C. tyrobutyricum

Cultures were initially grown on glucose, then subcultured into a 2 l medium supplemented with both sucrose and glucose in a 5 l stirred-tank fermentor. Batch cultivation experiments established that *C. tyrobutyricum* was capable of growing in basal medium supplemented with sucrose as the sole carbon source (Fig. 1A). Experiments with pairs of substrates revealed that glucose is preferred over sucrose as a substrate. Glucose was specifically depleted from the medium, concomitant with cell growth, whereas there was no perceptible utilization of sucrose (Fig. 1B). Cultures that were pregrown on sucrose also demonstrated preferential utilization of glucose when presented with both substrates, as sucrose utilization subsequently slowed down until the glucose had been exhausted from the medium (Fig. 1C). We therefore conclude that glucose can potentially regulate sucrose metabolism in *C. tyrobutyricum*.