Selective Fermentation of Xylose by a Mutant of *Tetragenococcus halophila* Defective in Phosphoenolpyruvate:Mannose Phosphotransferase, Phosphofructokinase, and Glucokinase

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*Tetragenococcus halophila* is a Gram-positive halophilic lactic acid bacterium used for soy sauce fermentation. We isolated a mutant, *T. halophila* 3E4, triply defective in phosphoenolpyruvate:mannose phosphotransferase, phosphofructokinase, and glucokinase. 3E4 selectively metabolized pentoses such as xylose and arabinose in the presence of hexoses such as glucose and galactose. We present here an example of the metabolic engineering of catabolite control.

Key words: phosphotransferase; phosphofructokinase; glucokinase; xylose fermentation; *Tetragenococcus halophila*

*Tetragenococcus halophila* (previously classified as *Pediococcus halophilus*) is a halophilic Gram-positive homo-fermentative lactic acid bacterium used for soy sauce fermentation.1,2 We studied this bacterium to establish mutants that can use pentoses such as xylose and arabinose even in the presence of high concentrations of glucose, to prevent the browning reaction of pentoses with amino acids in soy sauce, which would improve its shelf-life.1,2 Since fermented beverages and seasonings made from crops generally contain glucose as the major sugar component in addition to pentoses, the presence of a glucose obstacle for selective fermentation of pentoses due to glucose-mediated catabolite control. *T. halophila* has a phosphoenolpyruvate:mannose phosphotransferase system (man:PTS) that catalyzes both transport of glucose and its phosphorylation as a major glucose transporter.1,2 Mutations in the gene encoding the membrane component (EIIman) of man:PTS released glucose-mediated catabolite repression (EIIman- mutation designated as ptsM6).1,3 From the ptsM6 mutant X-160, we had isolated a mutant, M-13, defective in both EIIman and phosphofructokinase (EIIman- PK-; designated as ptsM6 pfk3).2,5 Although the double mutant M-13 hardly metabolized glucose, M-13 still took up glucose via a non-PTS carrier2 and the transported glucose was intracellularly phosphorylated by glucokinase (GK) resulting in accumulation of glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) (total amount >30 mM).2,6 Hexose phosphate (HXP) accumulation seems to be toxic for cell growth of M-13 because growth inhibition was observed when the glucose concentration in the medium was high (>30 mM).2,6 Interestingly, the glucokinase of *T. halophila* phosphorylated the glucose analog 2-deoxyglucose (dGlc) with ATP as a phosphorol donor3 in contrast to those of other homo-fermentative lactic acid bacteria such as *Lactococcus lactis*.3,6 The growth of M-13 was inhibited by dGlc (>15 mM) in the medium because large amounts of dGlc 6-P were accumulated intracellularly at toxic levels by a combination of the non-PTS glucose carrier and GK. Therefore, we derived from M-13 a spontaneous dGlc-resistant (50 mM) mutant, 3E4, defective in EIIman- PKF GK (EIIman- PKF- GK-), designated as ptsM6 pfk3 gk4.7 Comparison of the activities for glycolytic enzymes and man:PTS between 3E4 and related strains showed that 3E4 was a ptsM6 pfk3 gk4 mutant (Table 1). Glycolytic enzyme activities of cells grown in xylose [30 mM] BM-medium were measured by the methods previously described.1,2,7 3E4 had normal levels of the activities of the enzymes in the glycolytic pathway between aldolase and lactate dehydrogenase as the wild-type strain I-13 did (data not shown). Contrary to M-13, both [14C]fluorography8 and enzymatic analysis of glycolytic intermediates showed that cells of 3E4 scarcely accumulated G6P (<1 mM) even in the presence of 30 mM of glucose in the medium (Abe et al. unpublished results).

We further investigated the fermentation ability of 3E4 in a pentose-xylose mixed BM-MES medium9 (Fig. 1). Sugar components of the medium were measured by HPLC as described previously.1,1 The wild-type strain I-13 hardly metabolized galactose or pentoses, in particular xylose, in the presence of glucose due to catabolite repression. ptsM6 mutant X-160 fermented arabinose and small amounts of xylose and galactose. Since the ptsM6 pfk3 mutant M-13 did not grow well in this medium presumably due to the intracellular accumulation of toxic levels of HXP, only a small amount of arabinose was consumed. As expected, the ptsM6 pfk3 gk4 triple mutant 3E4 selectively metabolized xylose and...
Table 1. Activities of Man:PTS and Glycolytic Enzymes of T. halophila

<table>
<thead>
<tr>
<th>Enzymesa</th>
<th>Strainb</th>
<th>I-13</th>
<th>X-160</th>
<th>M-13</th>
<th>3E4</th>
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<tr>
<td>man:PTSb</td>
<td></td>
<td>5.8</td>
<td>0.6</td>
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<tr>
<td>Glucokinase</td>
<td></td>
<td>71.0</td>
<td>49.6</td>
<td>40.0</td>
<td>7.0</td>
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<tr>
<td>G6P dehydrogenase</td>
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<td>8.1</td>
<td>7.0</td>
</tr>
<tr>
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<td></td>
<td>1066.0</td>
<td>972.0</td>
<td>777.6</td>
<td>636.0</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td></td>
<td>107.0</td>
<td>132.0</td>
<td>2.6</td>
<td>7.0</td>
</tr>
</tbody>
</table>

a nmol/min/mg protein.
b nmol/min/mg dry cell wt (permeabilized cell).
c Cells were grown in BM-xylose (15 mM) glucose (15 mM) at 30°C. Enzyme activities were measured by the previously described method (1).

arabinose but scarcely consumed glucose or galactose.

Finally we examined the fermentation ability of 3E4 in moromi juice medium (MJ; soy sauce moromi filtered by Millipore membranes (0.45 μm)) and sugar consumption in the fermented MJ was measured by HPLC (Fig. 2). MJ contained approximately 380 mM of glucose, which inhibited the growth of M-13 by the intracellular accumulation of HXP. Thus, we eliminated M-13 from this examination. As expected, 3E4 selectively consumed xylene and arabinose in the presence of a large amount of glucose. After the fermentation, cells were removed from the MJ-medium by Millipore membranes (0.45 μm), and the filtered MJ fermented by 3E4 had a lighter color than those fermented by other strains (Abe et al. unpublished results). Even after storage at 30°C for 5 days, the filtered MJ fermented by 3E4 was a lighter color than those fermented by other strains (Abe et al. unpublished results). The application of the mutant to soy sauce fermentation is in progress.

Recently, we cloned the tetragenococcal xylose operon, the gene organization of which is: xylA (xylose isomerase)-xylB (xylulokinase)-xylE (xylose carrier). xylR, which is thought to function as the repressor of the xyl operon, was also found upstream of the xylA gene in the complementary sequence. Ducted XylIR protein contained a consensus sequence for binding catabolites of glucose such as G6P, which has been discovered in hexokinases in bacteria. The regulatory region of this operon contained both a putative binding site for XylIR and a consensus sequence, CRE (catabolite-responsive element), for binding of CcpA (catabolite control protein A). The speculated regulatory network of the xyl operon in T. halophila is shown in Fig. 3: (i) Catabolites of glucose such as fructose 6-phosphate (FDP) activate the HPr kinase, then the phosphorylated form of HPr(Ser) binds to CcpA mediating the dimerization of CcpA, CcpA with HPr(Ser)-P and a catabolite binds to the CRE sequence and represses expression of the xyl operon (catabolite repression); (ii) A catabolite such as G6P binds to XylIR, and activates it to the form capable of binding to the xyl opera-

![Fig. 1. HPLC Analysis of Sugar Consumption by T. halophila in BM-MES-Sugar Mixed Medium.](https://example.com/fig1)

Control (not fermented) contained glucose (60 mM), galactose (30 mM), xylose (30 mM), and arabinose (30 mM); Strains were I-13 (wild), X-160 (ptsM6), M-13 (ptsM6 pfk3), and 3E4 (ptsM6 pfk3 gk4). Fermentation was done for 5 days at 30°C.

![Fig. 2. HPLC Analysis of Sugar Consumption by T. halophila in MJ-Medium (Millipore Filtered Moromi).](https://example.com/fig2)

Control (not fermented) contained glucose (380 mM), galactose (24 mM), xylose (14 mM), and arabinose (13 mM); Strains were I-13 (wild), X-160 (ptsM6), and 3E4 (ptsM6 pfk3 gk4). Fermentation was done for 5 days at 30°C.
tor, resulting in repression of the xyl operon as reported for the xyl operon in *Bacillus subtilis* (antiinducer).\(^9\) (iii) Xylose itself binds to XylR, inactivating its release from the xyl operator included in the xyl operon. Based on this model, the release of catabolite repression in 3E4 is speculated to occur as follows: (i) The *ptsM* mutation in 3E4 lowers the accumulation of G6P; (ii) Intracellular free glucose transported by the non-PTS system is scarcely phosphorylated because of the *gk* mutation; (iii) FDP is hardly accumulated intracellularly due to the *pfk* mutation; (iv) Therefore, the low intracellular levels of glucose catabolites such as G6P and FDP cause the release of both the G6P-mediated antinducer phenomenon and the FDP-mediated catabolite repression, allowing cells to selectively ferment pentoses even in the presence of glucose (Fig. 3). The application of catabolite control mechanisms to the fermentation industry is an attractive approach. However, both molecular and physiological studies are further needed for the fine metabolic tuning of catabolite control in industrial strains.

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References