Functional Expression in \textit{Lactobacillus plantarum} of \textit{xylP} Encoding the Isoprimeverose Transporter of \textit{Lactobacillus pentosus}

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The \textit{xylP} gene of \textit{Lactobacillus pentosus}, the first gene of the \textit{xylPQR} operon, was recently found to be involved in isoprimeverose metabolism. By expression of \textit{xylP} on a multicopy plasmid in \textit{Lactobacillus plantarum} 80, a strain which lacks active isoprimeverose and D-xylose transport activities, it was shown that \textit{xylP} encodes a transporter. Functional expression of the \textit{xylP} transporter was shown by uptake of isoprimeverose in \textit{L. plantarum} 80 cells, and this transport was driven by the proton motive force generated by malolactic fermentation. \textit{XylP} was unable to catalyze transport of D-xylose.

\textit{Lactobacillus pentosus} is a facultative heterolactic bacterium which is characteristically found during the natural fermentation of vegetables. \textit{L. pentosus} develops predominantly in green olives, cucumbers, or cabbages in combination with yeasts and other heterolactic bacteria, such as species of the genus \textit{Leuconostoc} or \textit{Pediococcus} (4). In plant material fermentations, in which several microorganisms usually take part simultaneously and sequentially, the availability of nutrients is of crucial importance to the survival of one or more particular species of the microbial population. The ability to ferment the degradation products of the plant cell wall, a structure rich in polysaccharides, may be an important criterion of selection for the microflora adapted to growth on fermented vegetables. \textit{L. pentosus}, for instance, is capable of fermenting isoprimeverose [\(\alpha\)-D-xylopyranosyl-(1,6)-\(\alpha\)-D-glucopyranose], the major end product of xyloglucan hydrolysis. Recently, we reported that isoprimeverose metabolism in \textit{L. pentosus} involves the expression of an operon located within the \textit{xyl} regulon, whose expression is inducible by xylose (2). The operon comprises three genes: \textit{xylP}, encoding a putative permease; \textit{xylQ}, encoding a membrane-associated \(\alpha\)-xylosidase which is responsible for the hydrolysis of isoprimeverose into glucose and xylose; and \textit{xylR}, encoding a negative transcriptional regulator of the regulon. The xylose formed by the activity of the \(\alpha\)-xylosidase on isoprimeverose is further catabolized to xylulose 5-phosphate by D-xylose isomerase and D-xylulose kinase, encoded by the distal genes of the \textit{xyl} regulon, \textit{xylA} and \textit{xylB}. Disruption of \textit{xylP} did not abolish or reduce the ability of \textit{L. pentosus} to take up and metabolize D-xylose, suggesting that \textit{XylP} might be a transporter specific for the uptake of isoprimeverose (\(\alpha\)-xyloside) rather than a transporter of the monosaccharide. However, the \textit{ΔxylP} mutation exerted a polar effect on \textit{xylQ} expression. Therefore, a role for \textit{XylP} in isoprimeverose metabolism could not be assessed with certainty. A \textit{L. pentosus} mutant carrying a mutation of the \textit{xylP} gene without an effect on \textit{xylQ} expression was not obtained. For this reason, we have chosen another strategy to assess the role of \textit{XylP} in D-xylose and isoprimeverose uptake.

Knowledge about xyloside transporters in bacteria is scarce. So far, the only bacterial xyloside uptake system that has been characterized involves the product of the \textit{msiK} gene of \textit{Streptomyces lividans} (7). This gene encodes an ATP-binding protein required for the transport of cellobiose and xylobiose. The product of \textit{xylP} does not belong to the family of ATP-binding proteins but shows similarity to the galactoside-pentose-hexuronicide (GPH) translocators, a family of cation symporters which use the proton motive force (PMF) to drive the accumulation of di- or trisaccharides inside the cell (14). In this family, \textit{XylP} is most closely related to \textit{XynC}, a putative \(\beta\)-(1,4)-xylosidolate oligomeric (1,4)xylosidolate translocator encoded by a gene located in the \textit{xyl} regulon of \textit{Bacillus subtilis}. Its expression is induced by xylose (6). Although neither biochemical nor genetic evidence demonstrating a role for the product of \textit{XynC} in \(\beta\)-xyloside transport has been obtained, the similarity between \textit{XylP} and \textit{XynC} suggested that these two proteins might constitute a group of bacterial cation symporters specific for the uptake of xylosides.

To investigate the activity and substrate specificity of \textit{XylP} toward isoprimeverose and D-xylose, we attempted to express the \textit{xylP} gene in a bacterial strain which was deficient in D-xylose transport. Since radiolabelled isoprimeverose was not available, the most straightforward strategy for measurement of the accumulation of this \(\alpha\)-xyloside was to determine, after transport, the intracellular concentration of glucose liberated by hydrolysis of isoprimeverose by the \textit{L. pentosus} M3D353 \(\alpha\)-xylosidase. Therefore, to avoid degradation of the disaccharide during the transport experiment, it was important that the bacterial strain used in the isoprimeverose transport lacked all \(\alpha\)-xylosidase activity.

Expression of \textit{xylP} in \textit{L. plantarum} 80. The \textit{xylP} gene was cloned in a recently developed \textit{Lactobacillus} expression vector \textit{pLP503}(1) (15). The gene was expressed in \textit{Lactobacillus plantarum} 80 (16), a strain which is phylogenetically closely related to \textit{L. pentosus} (5) but lacks the ability to ferment both isoprimeverose and xylose. The lack of \textit{XylP} and \textit{xylQ} (\(\alpha\)-xylosidase) sequences in \textit{L. plantarum} 80 was confirmed by Southern hybridization of \textit{L. plantarum} 80 chromosomal DNA with \textit{XylP} or \textit{xylQ} probe under heterologous conditions (data not shown). For a number of \textit{Lactobacillus} strains, electrophor-
mation with plasmid DNA is inefficient and cloning of genes in *Lactobacillus* vectors requires a subcloning step in *Escherichia coli*. Thus, the construction of an *xylP* expression vector, pLPA6, was achieved by a multistep process (Fig. 1). Briefly, the *xylP* gene, which contained its original ribosome binding site (RBS) and that of the *xylQ* gene, was amplified from chromosomal DNA by PCR (using the ULTma enzyme [Perkin-Elmer]) and cloned into pTUT-MCS2 (9) downstream from a strong terminator (*T_{ldh}*, from *Lactobacillus casei* ATCC 393) generated an Apo site (underlined) downstream of the original *xylQ* RBS. The reverse primer (5′-TTCTAAGTACCAATTGAATTCAAGAAAGGGC-3′) used in the PCR amplification of *xylP* generated *BglII* and *KpnI* sites (underlined) and a stop codon (indicated in boldface) upstream of the original *xylP* RBS. The reverse primer (5′-TTACTAGTACCAATTGAATTCAAGAAAGGGC-3′) generated an *ApaI* site (underlined) downstream of the original *xylQ* RBS. The expression cassette of pLPA6 was derived from plasmid pLP503(t) (a broad-host-range expression vector) (15), which contains the constitutive promoter *P_{ldh}* of *L. casei* ATCC 393, the *β*-glucuronidase gene (*gusA*) from *E. coli*, and the terminator *T_{cbh}* of *L. plantarum* 80 (3). *bla*, *β*-lactamase (ampicillin resistance) determinant; *ermC*, erythromycin resistance determinant; ori−, origin of replication of pGEM; ori+, origin of replication of *Lactobacillus* plasmid pLP3537 (10); Rep, replication protein gene of pLP3537; 2*{T_{ldh}}*, two tandemly arranged transcription terminators of the *ldh* gene of *L. casei*.

**FIG. 1.** Strategy for the construction of the *Lactobacillus*-E. coli shuttle plasmid pLPA6(t) and the expression vector pLPA6. The forward primer (5′-TCTAGTACCAATTGAATTCAAGAAAGGGC-3′) used in the PCR amplification of *xylP* generated *BglII* and *KpnI* sites (underlined) and a stop codon (indicated in boldface) upstream of the original *xylP* RBS. The reverse primer (5′-TTACTAGTACCAATTGAATTCAAGAAAGGGC-3′) generated an *ApaI* site (underlined) downstream of the original *xylQ* RBS. The expression cassette of pLPA6 was derived from plasmid pLP503(t) (a broad-host-range expression vector) (15), which contains the constitutive promoter *P_{ldh}* of *L. casei* ATCC 393, the *β*-glucuronidase gene (*gusA*) from *E. coli*, and the terminator *T_{cbh}* of *L. plantarum* 80 (3). *bla*, *β*-lactamase (ampicillin resistance) determinant; *ermC*, erythromycin resistance determinant; ori−, origin of replication of pGEM; ori+, origin of replication of *Lactobacillus* plasmid pLP3537 (10); Rep, replication protein gene of pLP3537; 2*{T_{ldh}}*, two tandemly arranged transcription terminators of the *ldh* gene of *L. casei*.
TABLE 1. Accumulation of isoprimeverose

<table>
<thead>
<tr>
<th>Reaction no.</th>
<th>Uptake of isoprimeverose with protocol involving*</th>
<th>Intracellular glucose concn (mM) in†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L. plantarum 80/pLPA6</td>
</tr>
<tr>
<td></td>
<td>l-Malate</td>
<td>Isoprimeverose</td>
</tr>
<tr>
<td>1</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
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* +, uptake occurred; −, uptake did not occur. All experiments were performed in duplicate.
† Corresponding to 20 μg of xylene-grown L. pentosus MD353 membrane proteins.
‡ The transport reaction stopped at 2 min and at 10 min. All experiments were performed in duplicate.

ATCC 393) and circumvented instability of the expression vector in E. coli (15). Finally, the two Tn7p sequences were eliminated by digestion of the plasmid with NotI and religation, yielding pLPA6. As a result, a TAA stop codon located 10 nucleotides upstream of the original xylP RBS was in frame with the first few codons of the ldh gene and allowed translation of the native XylP protein. The cloning of the PCR fragment in the ApzI site of pTUT-MCS2 placed the original xylQ RBS 10 nucleotides upstream of the ATG codon of gusA, constituting a translation initiation site for this reporter gene. L. plantarum 80 was transformed with plasmid pLPA6 as described elsewhere (8), and the transformants were selected on M medium (8), and the transformants were selected for the uptake studies. L. plantarum 80 was transformed with plasmid pLPA6 and in wild-type L. plantarum 80. The strategy used to investigate the accumulation of nonradiolabelled isoprimeverose in L. plantarum was as follows. (i) L. plantarum 80 cells in the exponential phase of growth were harvested by centrifugation (5,000 × g, 4°C, 10 min), washed twice with 0.9% NaCl, and resuspended in KPM buffer (50 mM KH2PO4, 2 mM MgSO4), pH 4.5, at a concentration of 5 mg (dry weight)/ml. Subsequently, 500 μl of the cell suspension was incubated for 2 min at 37°C and energized with 50 mM L-malate. At a high concentration of l-malate (≥5 mM), L. plantarum cells take up this dicarboxylic acid by a low-affinity system which generates a large PMF (−160 mV) (11). Transport was initiated by adding isoprimeverose (purified from Tamarind seed xylglucan [2]) at a final concentration of 0.5 mM. After incubation, the reaction was quenched by addition of 2 ml of ice-cold 0.1 M LiCl and the cells were pelleted within a few seconds (15,000 rpm in a tabletop centrifuge). (ii) The pellet was resuspended in 200 μl of ice-cold KPM buffer (pH 6.5), and the cells were disrupted by shaking them at full speed (IKA-Vibrax-VXR; IKA-Labortechnik) for 1 h at 4°C with 50 mg of glass beads (0.1- to 0.3-mm diameter; Pertorps Analytical). After centrifugation (15,000 × g, 4°C, 15 min), the supernatant was boiled for 10 min at 100°C and denatured proteins were precipitated by centrifugation (15,000 × g, 4°C, 15 min). (iii) The supernatant was then incubated for 1 h with 20 μg of an L. pentosus MD353 membrane fraction containing α-xyllosidase (2). The glucose concentration in each of these samples was determined as described elsewhere (1), and the intracellular concentration of glucose was calculated by assuming an average intracellular volume of 3 μl/mg (dry weight).

In a control experiment, the α-xyllosidase was omitted to determine the concentration of glucose in L. plantarum cells. A second control experiment in which no l-malate was added prior to the transport reaction was carried out. The results are shown in Table 1. L. plantarum 80/pLPA6 took up and accumulated isoprimeverose against a concentration gradient. This uptake did not occur in the untransformed wild-type strain. Therefore, the product of the xylP gene is an isoprimeverose transporter. The uptake of isoprimeverose appeared to be driven by the PMF created by malolactic fermentation, since in the absence of l-malate the same amount of glucose (<0.2 mM) was found in transformed and untransformed bacteria (Table 1, reaction 1). The role of the PMF in the accumulation of isoprimeverose was further demonstrated when the extracellular pH was increased to 6.5, resulting in a strong decrease in both l-malate transport and the PMF resulting from it (11). Under these conditions, isoprimeverose could accumulate only to a concentration of 0.5 mM in L. plantarum 80/pLPA6 after 2 min. The uptake of isoprimeverose was linear for at least 2 min (Fig. 2).

α-Xylose transport in L. plantarum 80 cells harboring pLPA6 and in wild-type L. plantarum 80. We have also tested the ability of L. plantarum 80/pLPA6 and L. plantarum 80 wild-type cells to accumulate α-[U-14C]xylose (specific activity, 0.4 μCi/mmol; Amersham). The transport experiments were conducted as described above for the uptake of isoprimeverose. The
final concentration of l-xylose was 0.5 mM. In this case, the cells were rapidly filtered through glass fiber filters (GF/F; Whatman) after quenching and washed with 2 ml of ice-cold 0.1 M LiCl. No uptake or accumulation of [U-14C]-l-xylose by the two strains tested could be detected (data not shown). The use of radiolabelled xylose also enabled us to study the accumulation of pentose under different PMF-generating conditions, mediated by the fermentation of 5 mM glucose, added 5 min prior to the transport reaction. In this case, the reaction was performed at an extracellular pH of 6.5. Also under those conditions, xylose uptake by the two strains could not be detected (data not shown). These results demonstrate that XylP does not mediate uptake of xylose.

**Final conclusions.** This study has provided data confirming that the *L. pentosus* xylP gene encodes an isoprimeverose cation symporter. Under the conditions used for the transport experiment, the rate of isoprimeverose uptake was in the range of 3 nmol/min/mg (dry weight). It is possible that the rate of uptake may be higher under other conditions, especially at different pH values. Indeed, the extracellular pH can affect the activity of H⁺ symporters. The activity of the lactose H⁺ symporter LacS of *Streptococcus thermophilus*, for instance, is pH dependent, with an optimal activity at pH 6 and reduced activity at a lower or higher pH (13). Therefore, the extracellular pH of 4.5 used in the isoprimeverose transport assay, conditions required to generate a PMF via L-malate transport and metabolism, might not be an optimal pH for the activity of the XylP transporter. The transport of isoprimeverose by XylP was found to be dependent on the generation of a PMF, although the nature of the cation transported in symport with isoprimeverose is not yet known.

As mentioned above, XylP shows sequence similarity to the GPH family of translocators, especially to XynC of *B. subtilis*. Little is known about the XynC transporter, but Schmiedel and Hillen (17) have shown that *B. subtilis* expressing xynC could not take up D-xylose, indicating that XynC is not a D-xylose transporter. Similarly, our results show that XylP does not transport the monosaccharide. These observations provide the important finding that XylP and XynC are presumably transporters specific for the uptake of xyloides (with α and/or β linkages) but are not transporters of the pentose, although the designation of the GPH family had initially suggested otherwise (14).

Obviously, more-detailed studies of the XylP transporter could be conducted if radiolabelled xyloides were to become available. Recently, however, chromogenic substrate analogs have been used to assess uptake activity (12). Indeed, the design of chromogenic α/β-xyloides analogs may provide useful tools for the continued study of XylP and other xyloides transporters in bacteria.

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