Complementation of the Inability of Lactobacillus Strains To Utilize D-Xylose with D-Xylose Catabolism-Encoding Genes of Lactobacillus pentosus

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Lactobacillus strains are particularly known for their widespread application in industrial fermentation processes (11). Consequently, strain improvement of Lactobacillus spp. has always received considerable attention. During the past few years, major breakthroughs have been established in the genetic engineering of Lactobacillus strains. Vectors based on cryptic, endogenous Lactobacillus plasmids and antibiotic resistance genes have been constructed (1, 6, 13). Furthermore, methods for the introduction of (plasmid) DNA into almost every Lactobacillus species have been developed (1, 3, 6, 10, 13). In view of these recent achievements and the rapid increase in knowledge of gene expression in Lactobacillus spp. (2), construction by recombinant DNA techniques of Lactobacillus strains with improved or novel properties actually has come within reach. As the first step in the process of making a genetically modified Lactobacillus strain accepted by regulatory authorities, substitution of antibiotic resistance markers by food-grade markers seems obligatory. Up to now, food-grade selection systems for Lactobacillus spp. have not been described. Since only a few Lactobacillus species can utilize D-xylose as an energy source (8), we decided to exploit the potential of D-xylose fermentation as a food-grade selection marker for Lactobacillus spp.

Lactobacillus strains were routinely cultivated at 37°C in MRS broth (4) with 2% (wt/vol) glucose or 2% (wt/vol) D-xylose. For plating, MRS was solidified with 1.5% agar (Difco). Erythromycin was used at 5 μg/ml. Escherichia coli JM109 was used as the host strain for cloning in E. coli. Standard recombinant DNA techniques were carried out according to the methods of Sambrook et al. (14). Electroporation of Lactobacillus ATCC 393 and L. plantarum NCDO 1193 and isolation of Lactobacillus plasmid DNA were carried out as described elsewhere (12).

We have isolated two plasmids, pXHS50A and pXH37A (9), which together contain at least three genes involved in D-xylose catabolism in L. pentosus MD353. The genes are organized in a cluster on the chromosome (xyl cluster) in the order 5'-'xylR (encoding the presumed regulatory protein)-xylA (encoding D-xylose isomerase)-xylB (encoding D-xylose kinase)-3' (Fig. 1). To enable a functional analysis of the L. pentosus MD353 xyl cluster in Lactobacillus spp., the genes were inserted in the E. coli-Lactobacillus shuttle vector pLP3537 according to the following strategy. First, the 2.4-kb PstI-HindIII fragment of pXHS50A (encompassing xylR and the 5' part of xylA) was cloned in E. coli between the PsI and HindIII sites of pLP3537 (Fig. 1). Subsequently, the hybrid plasmid (pLP3537-1) was linearized with HindIII and ligated with the 3.7-kb HindIII fragment of pXH37A (containing the remaining part of xylA and xylB). We at first transformed E. coli with the ligation mixture. The resulting recombinant plasmid, however, appeared to be highly unstable.

FIG. 1. Cloning of three genes involved in D-xylose catabolism in a Lactobacillus vector. The genetic organization of the L. pentosus MD353 chromosomal DNA region containing xylR, xylA, and xylB (represented by thick arrows) is shown in the lower part of the figure. The number of amino acid residues (aa) in each protein is given in parentheses. The E. coli-Lactobacillus shuttle vector pLP3537, shown in the upper part of the figure, consists of pUC19 (thin line), an erythromycin resistance gene (ery), and a 2.3-kb plasmid (p353-2) of L. pentosus MD353.

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electroporation, the cell suspension was spread on MRS agar with D-xylose and bromocresol purple as an indicator for acid production. After 3 to 4 days of anaerobic incubation at 37°C, a few erythromycin-resistant transformants surrounded by a yellow halo were obtained. As shown in Fig. 2, in all of these transformants a plasmid (designated pLP3537-xyl) of the expected size and restriction enzyme pattern was present. The copy number of pLP3537-xyl is similar to that of pLP3537 (Fig. 2). When L. casei ATCC 393 and another non-D-xylose-fermenting strain, L. plantarum NCDO 1193, were retransformed with pLP3537-xyl isolated from L. casei ATCC 393, many (>10⁴ CFU/μg of DNA) erythromycin-resistant transformants were obtained that were all able to ferment D-xylose. Erythromycin-resistant transformants with pLP3537 or pLP3537-1, used as controls, did not produce enough acid from D-xylose for halo formation. Apparently, the D-xylose catabolism-encoding genes (xyl genes) present on pLP3537-xyl contain all of the information necessary to convert D-xylose into D-xylose-5-phosphate, an intermediate that can be further metabolized both by D-xylose-fermenting and non-D-xylose-fermenting Lactobacillus strains (5, 7). Plasmid DNA analysis of D-xylose-fermenting L. casei ATCC 393 and L. plantarum NCDO 1193 revealed that in all transformants pLP3537-xyl was present and structurally stable.

As can be deduced from the analysis of the growth rates of the different Lactobacillus strains in MRS medium and in MRS medium with glucose or D-xylose (Fig. 3), the efficiency with which Lactobacillus strains transformed with pLP3537-xyl can utilize D-xylose is comparable to that of the L. pentosus MD353 wild-type strain. In addition, the growth rate in the presence of D-xylose of L. pentosus MD353 or Lactobacillus strains transformed with pLP3537-xyl is always lower than that in the presence of glucose (Fig. 3).
In theory, there are several possible ways to improve the ability of *L. casei* ATCC 393 and *L. plantarum* NCDO 1193 transformed with pLP3537-xyl to ferment d-xylose. For example, improvement of the system may be achieved by the optimization of the expression of the *L. pentosus* MD353 xyl genes in the heterologous hosts and/or by the introduction of specific d-xylose transport function(s) in pLP3537-xyl. D-Xylose transport function(s) is not present on pLP3537-xyl (9).

Sequence analysis of the region upstream from *L. pentosus* MD353 xylR has indicated the presence of a putative d-xylose transport gene. Currently, experiments are being carried out aimed at the characterization and functional analysis of this gene and at the unraveling of the molecular mechanisms underlying the regulation of the expression of xyl genes in *L. pentosus* MD353. Together, these experiments will contribute to the development of a food-grade selection system for *Lactobacillus* spp. which is based on d-xylose fermentation.

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**REFERENCES**