4135-V

STUDIES ON INULIN-DEGRADING ENZYMES FROM LACTOBACILLI

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1. SUMMARY

Part of the surplus of agricultural crops in the Western world may serve as a source of raw material for industrial purposes. At present, attention is mainly focused at the processing of starch while limited use is made of other polymeric substances like for example the fructose polymer inulin. Large-scale availability of enzymes that convert inulin into compounds with a high added value like fructose and fructo-oligosaccharides may open new ways to crop usage. Some lactobacilli produce such enzymes. In this project, lactobacilli are screened for the presence of inulin-degrading enzymes. The gene(s) encoding such inulin-degrading enzymes will be cloned, characterized and used for the construction of *Lactobacillus* strains with improved properties with respect to the valorization of agricultural wastes and the manufacture of growth-promoting compounds.

2. INTRODUCTION

The surplus in Western societies of agricultural crops like cereals has prompted the search for alternative applications of crops that are commonly used. To date, most crops that serve as a source of raw material for industrial purposes are selected on the basis of their high starch content (*e.g.* potatoes). However, these and other crops also contain other polymeric substances that are presently insufficiently utilized. For example, large quantities of the fructose polymer inulin are found in cereals. About 70% of the dry weight of a young barley plant, for instance, consists of fructan.

The polymers inulin and levan, and products that can be obtained by partial or complete enzymatic hydrolysis of these polymers are of particular interest for biotechnological applications. Fructans as well as fructose offer attractive applications in the food industry, as such or as a starting material for the manufacture of other products (such as Ultra High Fructose Glucose Syrups, calory-poor sweeteners like mannitol, various organic acids, etc.). Also in the chemical industry fructans and fructose may become a useful starting material, a.o. in the production of compounds with a high added value like detergents and optically active compounds. Recently, the continuous production of gluconic acid and sorbitol from Jerusalem artichoke and glucose using an immobilized fructose-glucose

oxidoreductase from *Zymomonas mobilis* and inulinase was described (Kim and Kim, 1992).

2.1. Structure of inulin and levan

Both inulin and levan are fructose polymers, yet they are structurally different and are largely produced by different classes of organisms. Inulins which are primarily produced by plants are usually linear β-(2→1)-linked D-fructofuranose polymers with a terminal D-glucose unit and a MW of up to 6 kDa. Bacterial and fungal inulins - which are quite rare - are high-molecular weight (2 10^4 kDa) polyfructosides with varying degrees of branching at C6. Levans which are primarily produced by bacteria consist of high-molecular weight (10^5 kDa) branched molecules with predominantly β-(2→6) linkages, branched at C1. Furthermore, levan resembles inulin in it it also possesses a terminal glucose residue (Fuchs *et al.*, 1985).

2.2. Inulinase and levanase

There are two types of enzyme that can degrade fructans, hydrolases and transferases. Transferases produce difructose anhydrides from inulin via intramolecular transfructosylation. Hydrolases (inulinase and levanase) may split fructans either endo- or exohydrolytically, producing a series of oligofructans or only fructose, respectively. In yeasts, the enzyme responsible for the degradation of inulin is a non-specific fructosidase that is capable of hydrolysing both inulin and levan, as well as sucrose and raffinose. The enzyme liberates fructose molecules from sugars with β-(2→1)-linked fructose units at the terminal non-reducing end (Fuchs *et al.*, 1985). Yeast inulinase is distinguished from another fructosidase, invertase, which shows a low activity towards inulin. The S/I ratio (which indicates the relative activity towards sucrose and inulin) is commonly used to discriminate between the two enzymes. A low S/I ratio is indicative for an inulinase. Based upon kinetic evidence and on the lack of homology between the *N*-terminal amino acid sequences, Rouwenhorst *et al.* (1990) have concluded that yeast invertase and inulinase are distinct enzymes. Levanase is an enzyme produced by bacteria such as *Bacillus subtilis* and is involved in sucrose hydrolysis. The enzyme is secreted into the culture medium and hydrolyses inulin as well as levan (Kunst *et al.*, 1977).

2.3. Molecular genetics

Our knowledge on the regulation of expression of the genes involved in inulin/levan hydrolysis is limited. The gene (*sacC*) coding for levanase from *B. subtilis* has been cloned and sequenced. *SacC* is the distal gene of an operon containing four other genes, *levD*, *E*, *F* and *G* coding for a fructose-specific phosphotransferase system (Martin-Verstraete *et al.*, 1990). The expression of the levanase operon is inducible by fructose and subject to catabolite repression. A fructose-inducible promoter has been characterized 2.7 kb upstream of the levanase gene (Martin *et al.*, 1989). The upstream region of the levanase operon contains a positive regulatory gene called *levR* which is involved in the expression of the operon. *LevR* contains a domain thought to be specifically required for the formation of an open complex between σ54, the sigma factor of RNA polymerase and the promoter. Interestingly, the levanase operon in *B. subtilis* is transcribed from a -12, -24 promoter, which in Gram-negative bacteria is recognized by σ54 (Débarbouillé *et al.*, 1991).

The cloning and sequence of the inulinase gene (*INU*1) from *Kluyveromyces marxianus* has recently been described. *INU*1 displays a high level of evolutionary conservation with the invertase gene *SUC*2 from *Saccharomyces cerevisiae* (Laloux *et al.*, 1991).

2.4. *Lactobacillus* as a producer of inulinase

Lactobacilli are widely used in food and feed fermentations. They have a number of traits which make them highly suitable for the production of inulinase. For instance, lactobacilli are capable of preserving food and reducing the outgrowth of contaminating undesirable micro-organisms. Furthermore, lactobacilli have the GRAS (Generally Regarded As Safe) status and thus can be used for human and animal food fermentations. An outstanding property of a number of *Lactobacillus* species is their ability to very effectively utilize diverse carbon sources in rather extreme environments (low pH, anaerobic).

Recently, considerable progress has been made in the development of techniques for genetic modification of this group of organisms. Methods for the introduction and stable maintenance of DNA into *Lactobacillus* are routine now and can be applied to many *Lactobacillus* species. Both broad host-range and narrow host-range multi-copy plasmid vectors based on a variety of replicons have become available for the introduction and

expression of homologous and heterologous genes. Finally, methods have been developed to insert genes at specific sites of the chromosome allowing genes to be mutated at will (Pouwels *et al.*, 1992; Pouwels and Leer, 1993).

3. RESULTS AND DISCUSSION

3.1. Isolation and preliminary characterization of inulinase-producing lactobacilli

A number of lactobacilli were screened for the ability to degrade inulin (see Fig. 1). Eight potentially useful candidates were selected for further studies. These strains can grow on inulin as sole energy source. The growth characteristics of the strains in media containing inulin were compared with those in fructose (Fig. 2). Some strains can grow on inulin as fast as on fructose suggesting that inulinase activity is not rate-limiting. For a few strains it was observed that growth on inulin was much slower than on fructose. A possible explanation is that the release of fructose from inulin takes place at a low rate. It is not known whether *Lactobacillus* inulinases act as exo- and/or endo-hydrolysing enzymes. The presence of exo- and endoinulinases has been described for other microbes and may be responsible for the growth differences of the strains grown on inulin.

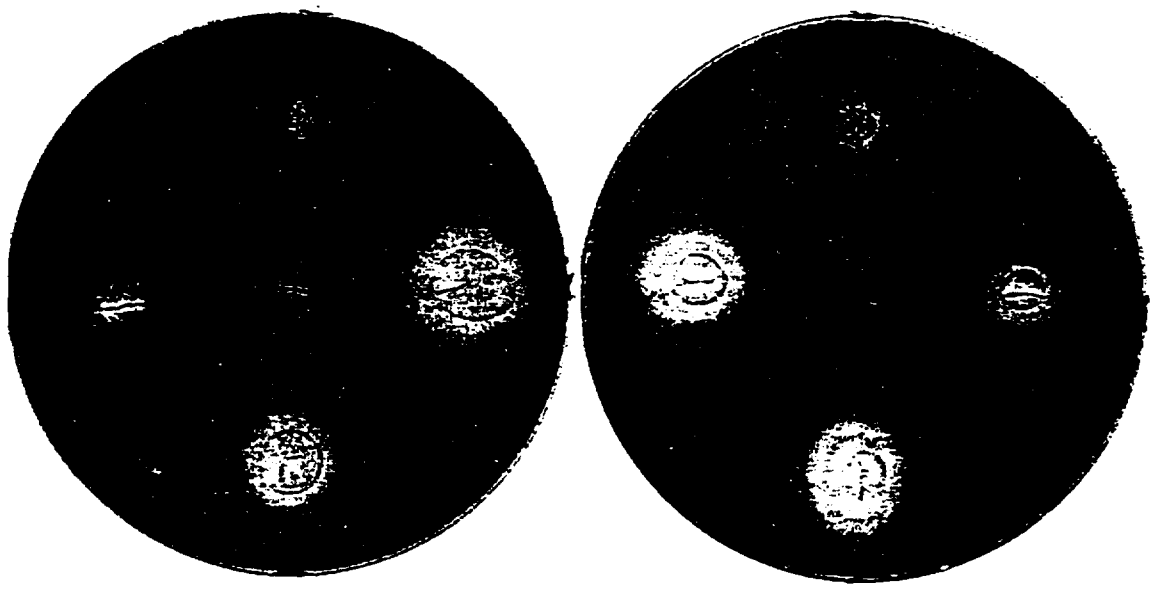

Fig. 1. Comparison of acid production on indicator plates containing glucose (left) and inulin (right) as the sole energy source. The controls are in the centre.

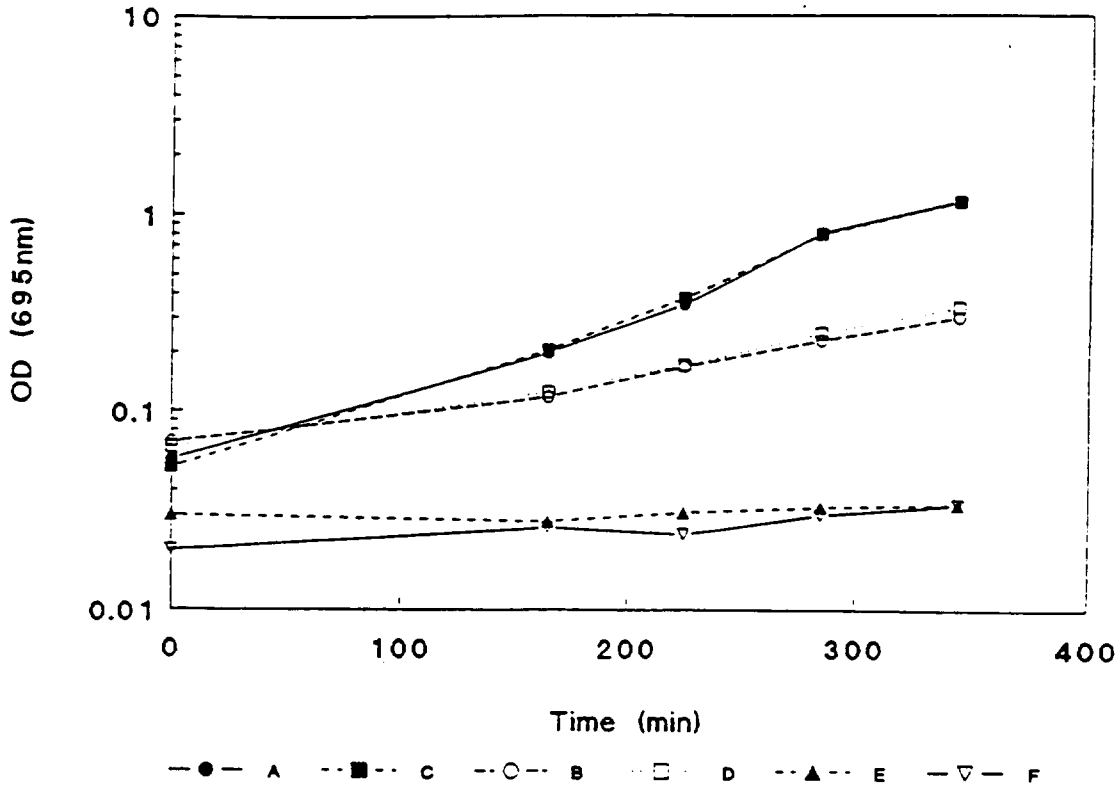

Fig. 2. Comparison of growth of four selected strains on inulin and fructose as the sole energy source. A, B, C and D: two strains of *L. pseudoplantarum* grown on fructose (A and C) and on inulin (B and D); E: *L. casei* ATCC 393, F: *L. plantarum* 80, both grown on inulin.

3.2. Cloning of *Lactobacillus* inulinase gene

Two strategies have been followed to clone the gene encoding inulinase from *Lactobacillus*. Firstly, based on a comparison of the nucleotide sequences of inulinase-encoding genes from *B. subtilis* and *K. marxianus* probes have been designed to facilitate the isolation of the gene with the Polymerase Chain Reaction (PCR) technique. Using chromosomal DNA of *L. pseudoplantarum* or other *Lactobacillus* strains as template no specific products could be synthesized. Secondly, we have attempted to identify the inulinase-encoding gene by Southern hybridization using the *B. subtilis* levanase gene as a probe (Wanker, 1992). As indicated in Fig. 3, two positive signals were obtained which differed in intensity. DNA corresponding to the hybridizing bands was isolated from the gels and cloned into pBR322 cut with the appropriate restriction enzymes. Transformant colonies were pooled and plasmid DNA isolated from the pools was analysed for the presence of the putative inulinase gene by Southern hybridization. Pools showing a positive signal were split into smaller pools, which led to the identification of

Fig. 3. Southern blot of *L. pseudoplantarum* chromosomal DNA after digestion with restriction enzymes. A part of the *B. subtilis* levanase gene was used as a probe.

individual transformants showing a positive signal in a Southern hybridization experiment. Restriction enzyme analysis of plasmid DNA of these transformants showed that the DNA fragment with the highest intensity in the Southern experiment had been cloned only. Experiments are in progress to also clone the DNA fragment with the weaker hybridization signal.

3.3. Sequence analysis

The position of the inulinase gene on the cloned fragment was determined by restriction enzyme analysis and hybridization with the *B. subtilis sacC* fragment as a probe. DNA sequence analysis of two regions of the cloned fragment revealed the presence of open reading frames. A comparison of the deduced amino acid sequence of the putative inulinase of *L. pseudoplantarum* with that of the *B. subtilis* levanase shows that the two proteins display considerable similarity (Fig. 4). Further research is necessary to unequivocally identify the nature of the cloned gene.

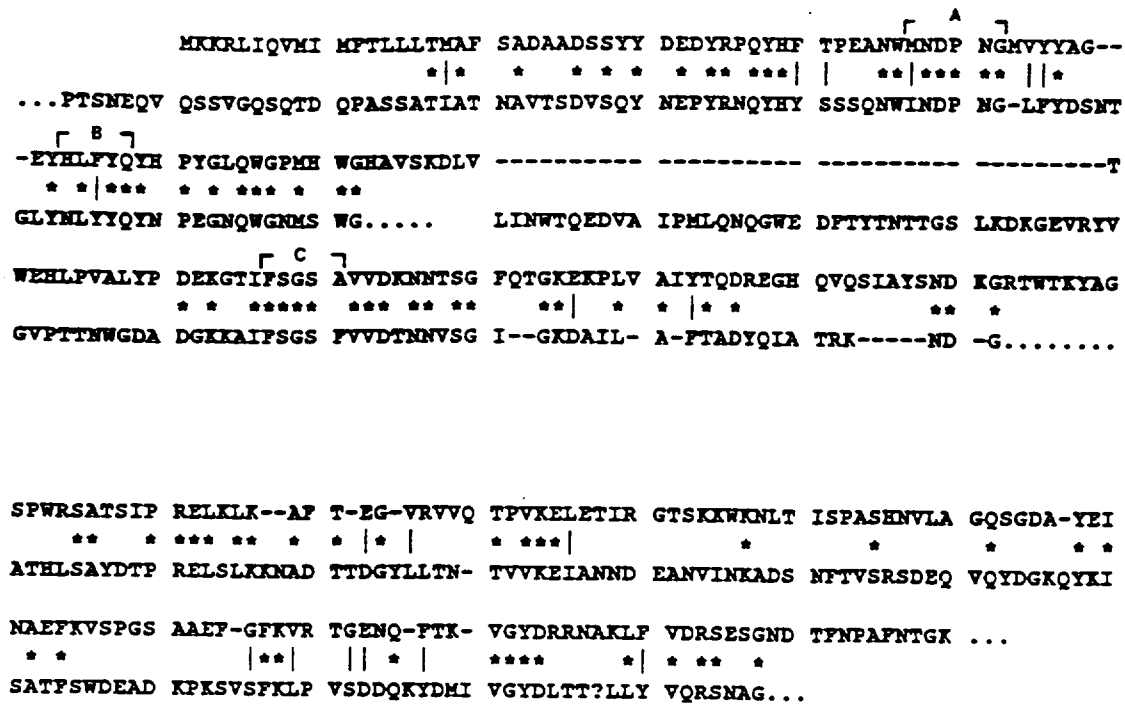

Fig. 4. Comparison of the amino acid sequence of the levanase gene from *B. subtilis* (upper rows) with that of the putative inulinase gene of *L. pseudoplantarum*. Asterisks and bars indicate identical and similar amino acids, respectively. Well-conserved regions are boxed.

3.4. Expression of *B. subtilis* levanase gene in *Lactobacillus*

In collaboration with Drs Erich Wanker and Helmut Schwab (Technological University Graz, Austria), experiments have been carried out aimed at the expression of the inulinase gene from *B. subtilis* in *Lactobacillus*. Since in *B. subtilis* the inulinase-encoding gene, *sacC*, comprises the promoter distal part of an operon, the cloned DNA fragment lacked promoter sequences (Martin-Verstraete *et al.*, 1990; Wanker, 1992). To drive the expression in *Lactobacillus*, the *sacC* gene was placed under the control of the *Escherichia coli tac* promoter on the multi-copy shuttle vector pLP3537, which can replicate both in *E. coli* and *Lactobacillus* (Posno *et al.*, 1991). To control expression of the *tac* promoter, the gene encoding the *lac* repressor, $lacI^q$, was introduced into the vector. After transformation of *L. casei* or *L. plantarum* with this vector, inulinase activity could be detected in the culture fluid of stationary-phase cultures. Preliminary data indicate that also in the absence of IPTG, the inducer of the *tac* promoter, inulinase activity could be observed. This result might indicate that the $lacI^q$ gene is not expressed in *Lactobacillus* or that the repressor

formed is not active in *Lactobacillus*. Our results demonstrate for the first time that lactobacilli can be genetically engineered to express the *B. subtilis* inulinase. They also indicate that the *tac* promoter is active in lactobacilli.

4. LITERATURE

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