Production of secreted guar α-galactosidase by Lactococcus lactis

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Abstract A plant α-galactosidase gene was inserted in the expression vector pGKV259. The resulting plasmid pGAL2 consisted of the replication functions of the broad-host-range lactococcal plasmid pWV01, the lactococcal promoter P59, and the DNA sequences encoding the α-amylase signal sequence from *Bacillus amyloliquefaciens* and the mature part of the α-galactosidase from *Cyamopsis tetragonoloba* (guar). *Lactococcus* cells of strain MG1363 harbouring this vector produced the plant α-galactosidase and secreted the enzyme efficiently as judged by Western blotting and activity assays. Expression levels of up to 4.3 mg extracellular α-galactosidase g (dry weight) of biomass^{-1} were achieved in standard laboratory batch cultures. The α-galactosidase produced by *Lactococcus* was active on the chromogenic substrate 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside, the trisaccharide raffinose and on the galactomannan substrate, guar gum.

Introduction

Specific commercial applications may require efficient secretion of heterologous gene products from the producing organism. *Lactococcus lactis*, a gram-positive

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bacterium traditionally used in a variety of dairy fermentations, is a genetically easily accessible organism with GRAS status (generally recognized as safe). Therefore, *L. lactis* is an attractive organism for the safe production of foreign proteins. In *L. lactis*, the Sec-dependent secretory pathway has been studied to some extent with respect to the secretion of heterologous proteins. Several homologous signal sequences have been used to secrete the bovine prochymosin (Simons et al. 1992), the *Bacillus stearothermophilus* α-amylase (Simons et al. 1990), the *Bacillus licheniformis* α-amylase (Pérez-Martínez et al. 1992), the *Escherichia coli* β-lactamase (Pérez-Martínez et al. 1992; Sibakov et al. 1991) and the tetanus fragment C of *Clostridium tetanus* (Wells et al. 1993a). The *Bacillus subtilis* neutral protease and the *Streptococcus equisimilis* streptodornase were secreted from *Lactococcus* using their native signal sequences (van de Guchte et al. 1990; Wolinowska et al. 1991).

The α-galactosidase (EC 3.2.1.22) of the legume *Cyamopsis tetragonoloba* (guar) is produced in the endosperm by the aleurone cells (Meier and Reid 1982) and is subsequently secreted into a reserve polysaccharide layer (galactomannan) during seed germination. The guar α-galactosidase has the unique and commercially attractive property of being able to release galactose from galactomannan, resulting in a galactomannan with improved gelling properties (Critchley 1987). The gene encoding guar α-galactosidase has been cloned (Overbeeke et al. 1989) and to study the enzyme in more detail in prokaryotes it was expressed and secreted by *B. subtilis* DB104 using the *B. amyloliquefaciens* α-amylase signal peptide. Although this protease-deficient strain efficiently secreted the α-galactosidase in the exponential growth phase, residual protease activity caused a considerable decrease in the enzyme activity upon prolonged cultivation (Overbeeke et al. 1990).

Substantial loss of enzyme activity due to proteolytic breakdown of (heterologous) gene products in *L. lactis*

has not been reported. Furthermore, the facultative anaerobic *L. lactis* allows simple cultivation conditions and tools for its genetic manipulation are available. Therefore, *L. lactis* could serve as an attractive alternative for the efficient production and secretion of heterologous proteins. In the present work, we demonstrate that the *L. lactis* Sec-dependent secretory pathway allows the efficient secretion of an active plant α-galactosidase using a heterologous prokaryotic signal peptide. The enzyme is produced constitutively in batch cultures and is not degraded during the stationary growth phase.

Materials and methods

Bacterial strains, plasmids and growth conditions

The *Bacillus* ssp. strain DB104 (*his nprR2 nprE18 aprA3*) (Kawamura and Doi 1984) and 1012 (*leuA8 metB5*) (Saito et al. 1979), and the *L. lactis* ssp. strain MG1363 (*lac prtP prtM*) (Gasson 1983) were used as hosts. Plasmid pUR2601 has been described before (Overbeeke et al. 1990) and is a derivative of the *Bacillus* vector pLP608 (Williams et al. 1981). It confers resistance to kanamycin and contains a fusion of the SPO2 promoter, a consensus ribosome-binding site, the α-amylase signal sequence from *B. amyloliquefaciens* (Palva et al. 1981) and specifies the mature guar α-galactosidase (Overbeeke et al. 1990) (Fig. 1). Plasmid pGKV259 has been constructed by van der Vossen et al. (1987). It is a broad-host-range vector of lactococcal origin and carries an erythromycin-resistance gene and the strong lactococcal promoter P59 (Fig. 1). Plasmid pGAL2 is described in the text. *B. subtilis* was grown in TY broth (Rottlander and Trautner 1970) or on TY broth solidified with 1.5% agar. In *B. subtilis* protoplast transformation, DM3 was used as the plating medium (Chang and Cohen 1979). For Western blot analysis *B. subtilis* (pUR2601) was grown to A_{660} of 0.5 in minimal medium (Smith et al. 1983). *L. lactis* was grown in glucose M17 (GM17) broth (Terzaghi and Sandine 1975), or on GM17 broth solidified with 1.5% agar. Sucrose (0.3 M) was added to the M17-based medium to stabilize electrotransformed *L. lactis* cells osmotically. For HPLC and Western blot analysis *L. lactis* was grown in whey-permeate-based medium (de Vos et al. 1989). Kanamycin and erythromycin were used at a concentration of 5 μg/ml for *B. subtilis* and *L. lactis*.

DNA manipulation and transformation

Plasmid DNA was isolated from *B. subtilis* and *L. lactis* as described by Birnboim and Doly (1979). The lysis step was carried out at 37°C. For *L. lactis*, mutanolysin (Sigma Chemical Co., St. Louis, Mo.) was added to the lysis solution to a final concentration of 75 U/ml. Plasmid DNA was prepared by CsCl/ethidium bromide centrifugation as described before (Ish-Horowicz and Burke 1981). Restriction enzymes, Klenow enzyme and T4 DNA ligase were purchased from Boehringer (Mannheim, Germany) and used according to the instructions of the supplier. Protoplasts of *B. subtilis* were prepared and transformed using the procedure of Chang and Cohen (1979). *L. lactis* was electrotransformed as described before (Leenhouts et al. 1990) using a BioRad Gene Pulser (BioRad Laboratories, Richmond, Calif.).

Biomass determination

Biomass dry weight was determined by drying the twice-washed samples for 16 h at 60°C.

Assays for α-galactosidase activity

For the detection of *L. lactis* transformants producing active α-galactosidase, the chromogenic substrate 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside (X-α-Gal; Boehringer) was incorporated in the agar plates as described by Tubb and Liljeström (1986). The activity of α-galactosidase in culture supernatants and cell-free extracts was determined by assaying the degradation of *p*-nitrophenyl α-D-galactopyranoside (Np-Gal; Sigma Chemical Co.) at 37°C, essentially as described by Overbeeke et al. (1990). Cell-free extracts were prepared as described before (van de Guchte et al. 1992). To 900 μl 22 mM Np-Gal in 0.1 M sodium acetate (pH 5.0), 100 μl (diluted) sample was added. The reaction was stopped after 10 min by the addition of 2 ml 10% (w/v) sodium carbonate, followed by the determination of the A_{405}. The α-galactosidase activity was calculated by using the absorption coefficient of *p*-nitrophenol at 405 nm (18.4 cm²/μmol), and 1 U was defined as the hydrolysis of 1 μmol substrate/min under the assay conditions. The specific activity of α-galactosidase, 100 U mg protein^{-1}, has been determined before (Fellinger et al. 1991).

The activity of α-galactosidase produced by *L. lactis* on raffinose was determined as follows. *L. lactis* with and without pGAL2 were grown in whey-permeate-based medium. After overnight growth the cultures were centrifuged and 10 ml supernatant of each culture was dialysed at 4°C for 28 h against a total volume of 10 l distilled water to remove all sugars. The samples were dried in a SpeedVac concentrater (Savant Instruments, Farmindale, N.Y.) and the pellet was dissolved in 1 ml 0.1 M sodium acetate pH 5.0. After the addition of 100 μl concentrated supernatants (with 2 μg α-galactosidase for the pGAL2-containing culture) to 900 μl 17 mM raffinose (Sigma Chemical Co.) in 0.1 M sodium acetate pH 5.0, the samples were incubated for 1 h at 37°C followed by HPLC analysis (BioRad) using a 30-cm organic-acid column (HPX-87H). Samples were eluted with 5 mM sulphuric acid and the sugar moieties were detected according to the refraction-index method.

Activity on guar gum was determined by measuring the amount of galactose liberated after incubation with α-galactosidase, using the raffinose test kit 428176 (Boehringer). For this purpose an amount of guar gum equivalent to 20 μmol total sugar was dissolved in 0.1 M sodium acetate pH 4.7 and incubated for 25 h at 37°C with 1 μg/ml α-galactosidase.

SDS-PAGE and Western blotting

Protein samples were subjected to 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970), using the BioRad Protean II minigel system. Proteins were transferred onto BA85 nitrocellulose (Schleicher & Schüll Inc., Keene, N.H.) essentially as described by Towbin et al. (1979). Antigens were detected by using 5000-fold-diluted guar-α-galactosidase-directed antisera (Overbeeke et al. 1990). and alkaline-phosphatase-conjugated goat anti-(rabbit immunoglobulins) (Promega, Madison. Wis.) according to the manufacturer's instructions.

Results

Construction of the expression vector pGAL2

The broad-host-range lactococcal vector pGKV259 was used to express the in-frame fusion of the *B. amyloliquefaciens* α-amylase signal sequence and the mature *C. tetragonoloba* α-galactosidase. For this purpose a 0.6×10^3-base (0.6-kb) fragment, containing

Fig. 1 Schematic respresentation of the construction of pGAL2. Only relevant restriction enzyme sites are shown. P_{59} constitutive lactococcal promoter, P_{SP02} B. subtilis phage SP02 promoter, cat chloramphenicol acetyltransferase gene of B. pumilus, α-gal α galactosidase gene of C. tetragonoloba, Em^r erythromycin-resistance gene, Km^r kanamycin-resistance gene. □ Ribosome-binding site, ■ signal sequence of B. amyloliquefaciens
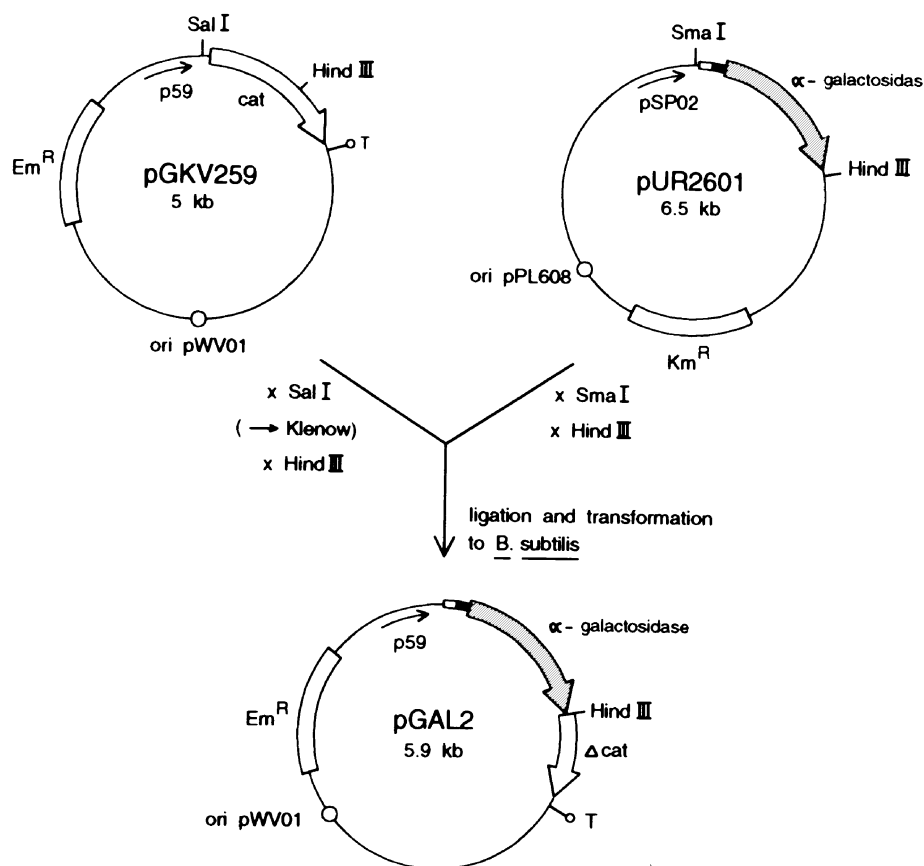
most of the cat86 gene, was deleted from pGKV259 by digestion with SalI and HindIII. The SalI site of the vector was treated with Klenow enzyme to fill-in the recessed ends. The 1.5-kb SmaI-HindIII α-galactosidase fragment was isolated from the B. subtilis vector pUR2601 and ligated to the lactococcal vector (Fig. 1). Since it has been shown before that B. subtilis is able to secrete the active plant enzyme, the ligation mixture was used to transform B. subtilis 1012. Erythromycin-resistant transformants were screened for their inability to grow on chloramphenicol. Plasmid DNA was isolated from chloramphenicol-sensitive transformants, and was analysed with restriction enzymes. Transformants from which the plasmids showed the expected restriction patterns were assayed with Np-Gal for the presence of α-galactosidase in the culture medium of exponentially growing cells. A transformant showing all the expected characteristics, ie. Em^r, Cm^s and αGal$^+$, was used to isolate plasmid pGAL2.

Transformation of pGAL2 to L. lactis

The Em^r colonies obtained with L. lactis MG1363 transformed with pGAL2 were screened for α-galactosidase activity by transfer to an agar plate containing X-α-Gal as an indicator (blue colour). After overnight growth, all transferred colonies were blue, whereas strain MG1363 carrying pGKV259 remained white on such plates. This result suggested that an active α-galactosidase was produced by L. lactis MG1363(pGAL2).

Western blot analysis

Samples taken from the growth medium and from a cell extract of an overnight-grown culture were subjected to Western blot analysis to determine the localization of guar α-galactosidase and to examine whether L. lactis processed the precursor correctly by comparison to samples taken from an exponentially growing B. subtilis DB104(pUR2601) culture. In B. subtilis correct processing of the enzyme has been demonstrated (Overbeeke et al. 1990). The samples taken from B. subtilis and L. lactis culture media showed one dominant band of approximately 40 kDa (Fig. 2; lanes 2 and 3 respectively), which is in agreement with the molecular mass of 39.7 kDa calculated from the amino acid sequence of the mature non-glycosylated enzyme (Overbeeke et al. 1989). The weak bands with lower molecular masses are most likely the result of proteolytic breakdown. The cell extracts of the B. subtilis and L. lactis cultures (Fig. 2, lanes 1 and 4 respectively)
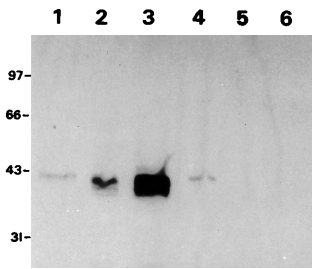
Fig. 2 Western blot analysis for the presence of α-galactosidase. Lanes: *1* cell extract of 200 μl exponentially growing ($A_{660} = 0.5$) *B. subtilis* (pUR2601) culture, *2* 10 μl culture medium of exponentially growing *B. subtilis* (pUR2601), *3* 100 μl culture medium of *L. lactis* (pGAL2) overnight culture, *4* cell extract of 100 μl *L. lactis* (pGAL2) culture grown overnight, *5* 100 μl culture medium of *L. lactis* (pGKV259) overnight culture, *6* cell extract of 100 μl *L. lactis* (pGKV259) culture grown overnight

both show the unprocessed form of the α-galactosidase, which migrated as a single band with a slightly higher molecular mass than the band in the culture medium. It is concluded from this experiment that *L. lactis* produced the α-galactosidase in a non-glycosylated form, secreted the enzyme efficiently and, most likely, processed it correctly.

Activity of guar α-galactosidase produced by *L. lactis* on raffinose and guar gum

The activity of the α-galactosidase produced by *L. lactis* on the trisaccharide raffinose was tested as described in Materials and methods. As a negative control *L. lactis* without pGAL2 was used. The quantities of liberated galactose are listed in Table 1 and show that the enzyme produced by *L. lactis* (pGAL2) is active on raffinose. Since *L. lactis* is able to grow on galactose we tested the growth capacity of strain *L. lactis* (pGAL2) in M17 medium with raffinose as the sole carbon source. However, significant growth of this strain was not observed (results not shown).

L. lactis (pGAL2) α-galactosidase preparations (see Materials and methods) were also tested for activity on guar gum. The non-glycosylated enzyme isolated from *B. subtilis* (pUR2601) has been shown to be active on

Table 1 Activity of *L. lactis*-produced α-galactosidase on raffinose and guar gum expressed as galactose released (*NT* not tested)

Enzyme source	Galactose released (nmol/ml) from:	
	Raffinose	Guar gum
L. lactis	0	0
L. lactis (pGAL2)	4×10^3	198
B. subtilis (pUR2610)	NT	204

this substrate in the same way as the plant-derived enzyme (Overbeeke et al. 1990) and was used in this assay as a positive control. The results (Table 1) show that the activity of the enzyme produced by *L. lactis* is comparable to that of *B. subtilis*.

α-Galactosidase expression level in *L. lactis*

L. lactis MG1363 (pGAL2) was grown overnight in liquid M17 medium containing 1% glucose and erythromycin. The culture was diluted 100-fold in the same medium and samples were taken as a function of time to measure the culture density, biomass and the amount of α-galactosidase in the culture medium (Fig. 3). The highest productivity level, 6.2 mg α-galactosidase g biomass^{-1}, was calculated at the 5-h assay time (data not shown), when the total amount of α-galactosidase produced was 4.7 mg/l with a biomass of 750 mg dry weight/l. A sharp decrease in the production occurred in the late exponential phase between the 5 and 6-h assay times, when the biomass still increased considerably by 450 mg/l, but the amount of α-galactosidase by only 0.5 mg/l. After 8 h of growth the culture was in the stationary phase with an A_{660} of 3.4 and had reached a level of 5.9 mg/l active α-galactosidase in the growth medium. Prolonged incubation for a subsequent 16 h resulted in a slight increase in the production level to 6.4 mg/l.

The intracellular amount of α-galactosidase was measured at two assay times. The α-galactosidase activity in cell extracts was 0.03 mg/l and 0.04 mg/l for the 4-h and 8-h assay times respectively. This result shows that more than 98% of the enzyme activity was present in the culture medium, indicating a very efficient secretion of the plant enzyme by *L. lactis*.

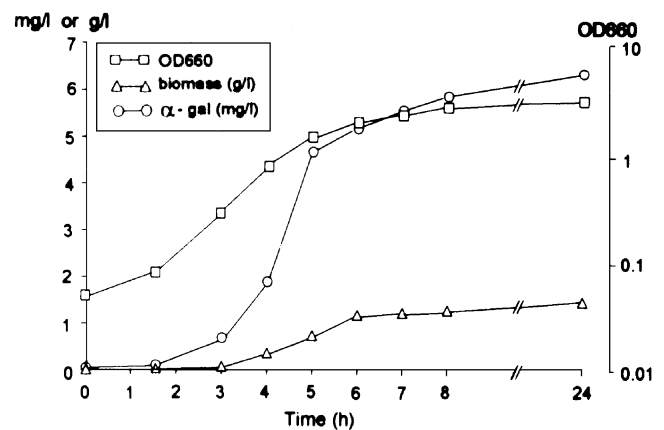In these batch cultures *L. lactis* reached a maximum biomass level after 24 h of 1.5 g/l with a corresponding

Fig. 3 Growth and α-galactosidase production of *L. lactis* (pGAL2) in a 1-l batch culture in M17 medium with 1% glucose

productivity of 4.3 mg extracellular α-galactosidase g biomass^{-1}.

Discussion

The successful use of a heterologous prokaryotic signal peptide to secrete a eukaryotic enzyme is the first example of such an approach in *L. lactis* and demonstrates the feasibility of exploiting the *L. lactis* Sec-dependent secretory pathway for the production of protein from vegetable origin. The processing of the α-amylase signal peptide was, most likely, correct and complete as judged by activity assays and by comparison in Western blots to α-galactosidase produced by *B. subtilis*. The unprocessed form of the enzyme, present in the cell extracts, represented less than 2% of the total amount produced. The maximum production level in *L. lactis* batch cultures was 4.3 mg secreted α-galactosidase g biomass^{-1}, which is 2.5-fold higher than that achieved in *B. subtilis*. Laboratory-scale fermentation processes were required for *B. subtilis* to suppress proteolytic activity to achieve a maximal production level of 1.7 mg g biomass^{-1} (Overbeeke et al. 1991). Expression levels in continuous culture systems of the guar α-galactosidase gene cloned into *Saccharomyces cerevisiae* (Verbakel 1991) and *Hansenula polymorpha* (Fellinger et al. 1991) are approximately seven to ten fold higher than in *B. subtilis* respectively (Giuseppin et al. 1993). However, in the *B. subtilis* and yeast systems high-copy-number (integration) vectors and strong inducible promoters were used, whereas in *L. lactis* the expression vector was of the low-copy-number type carrying a constitutive promoter. Therefore, we assume that the production level in *L. lactis* may be increased several fold by using other vectors and promoters. For this purpose the high-copy-number pIL253-based vectors (Simon and Chopin 1988) may be suitable. Alternatively, Campbell-type integration vectors, which give stable multiple-copy integrations, can be used (Leenhouts and Venema 1992). Inducible promoters from the *L. lactis* lac operon, in combination with the T7-polymerase system (Wells et al. 1993a), or the inducible lactococcal phage rlt promoters (R. Nauta, unpublished results) could be used to optimize expression.

The relatively low absolute yield in *L. lactis* of 6.4 mg α-galactosidase/l is largely the result of the low biomass levels (1.5 g/l) obtained in batch cultures. Much higher biomass levels should be obtainable in continuous cultures. However, the performance of *L. lactis* in continuous cultures has not been studied in detail. Therefore, additional growth studies are required to determine the performance of *L. lactis* as a production organism of heterologous proteins.

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