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Development of an Amylolytic *Lactobacillus plantarum* Silage Strain Expressing the *Lactobacillus amylovorus* α-Amylase Gene

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An amylolytic *Lactobacillus plantarum* silage strain with the starch-degrading ability displayed by *Lactobacillus amylovorus* was developed. An active fragment of the gene coding for α-amylase production in *L. amylovorus* was cloned and integrated into the chromosome of the competitive inoculant strain *L. plantarum* Lp80 at the *cbh* locus. The α-amylase gene fragment was also introduced into *L. plantarum* Lp80 on an autoreplicative plasmid. Both constructions were also performed in the laboratory strain *L. plantarum* NCIB8826. All four recombinant strains secreted levels of amylase ranging from 23 to 69 U/liter, compared with 47 U/liter for *L. amylovorus*. Secretion levels were higher in *L. plantarum* NCIB8826 than in *L. plantarum* Lp80 derivatives and were higher in recombinant strains containing autoreplicative plasmids than in the corresponding integrants. The *L. plantarum* Lp80 derivative containing the *L. amylovorus* α-amylase gene fragment integrated into the host chromosome secreted α-amylase to a level comparable to that of *L. amylovorus* and was stable over 50 generations of growth under nonselective conditions. It grew to a higher cell density than either the parent strain or *L. amylovorus* in MRS medium containing a mixture of starch and glucose as the fermentable carbohydrate source. This recombinant α-amylolytic *L. plantarum* strain would therefore seem to have considerable potential as a silage inoculant for crops such as alfalfa, in which water-soluble carbohydrate levels are frequently low but starch is present as an alternative carbohydrate source.

Silage making is an important method of crop preservation throughout the major agricultural regions of the world, with grasses, corn, and legumes such as alfalfa being the crops most frequently ensiled. Silage fermentation depends on lactic acid bacteria present on the crop fermenting water-soluble carbohydrates to lactic and acetic acids. The resulting low pH combined with the toxicity of the undissociated acids restricts further microbial activity and ensures good preservation. Low numbers of homofermentative acid-tolerant lactic acid bacteria, in the range 10^2 to 10^4 per g of fresh material, are frequently found on the fresh crop (6, 31), and the addition at ensiling of large numbers of lactic acid bacteria of this type, typically strains of *Lactobacillus plantarum* at a dose of 10^6/g, has been shown to aid preservation (10, 20, 27). However, bacterial inoculants are of little benefit when water-soluble carbohydrate levels in the fresh crop are insufficient to allow the production of enough lactic acid to ensure preservation, a situation frequently encountered in the ensiling of alfalfa (23, 31). However, alfalfa contains significant quantities of starch (23), which, though unavailable to most conventional bacterial inoculants, could be used as an additional source of carbohydrate by an amylolytic inoculant strain. *Lactobacillus amylovorus* was previously identified as possessing an amylolytic activity suitable for application in silage fermentation, with an enzyme pH optimum of 5.5 and a temperature range extending to 55°C (8).

We therefore endeavoured to design an amylolytic silage inoculant based on *L. amylovorus* by transferring its α-amylase gene to a competitive inoculant strain of *L. plantarum* by gene cloning techniques. In order to conform with regulations governing the release of genetically manipulated organisms into the environment, such an amylolytic recombinant *L. plantarum* strain would have to contain the heterologous amylase gene stably integrated into the host chromosome and free of any residual vector sequences. Previous studies indicate that the *L. plantarum* conjugated bile acid hydrolase gene (*cbh*) represents a suitable locus for targeting such an integration. Conjugated bile acid hydrolase is involved in cell metabolism in the intestinal environment only (3, 4), and disruption of this gene would not be expected to affect *L. plantarum* growth rates in silage.

Leer et al. demonstrated that the chromosomal *cbh* gene in *L. plantarum* Lp80 was a suitable site for targeting integration of heterologous genes by substituting an interrupted *cbh* gene containing a chloramphenicol resistance gene for the chromosomal *cbh* gene by homologous double-crossover recombination (18). The resulting strain had a Cbh^- Cm^+ phenotype, which was stably maintained for more than 100 generations of growth under nonselective conditions. It was therefore decided to integrate the *L. amylovorus* amylase gene at the *cbh* locus in the *L. plantarum* chromosome. Previous attempts to construct an amylolytic *L. plantarum* strain by chromosomal integration of an amylase gene of *Bacillus* origin proved unsuccessful, as the recombinant strain secreted negligible quantities of amylase (5, 13, 26), perhaps because of incompatibility between expression and/or secretion signals of *Bacillus* origin and the mechanisms for enzyme production and secretion in *L. plantarum*. Such problems are unlikely to occur if *L. amylovorus* is used as the source of an amylase gene, as expression and

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TABLE 1. Plasmids and bacterial strains

Plasmid or strain	Genotype or relevant features	Origin or reference
Plasmids		
pGK13	Shuttle vector, *E. coli-Lactococcus lactis*; Em^r Cm^r	17
pGIP73	Shuttle vector, *E. coli-L. plantarum*; derivative of pJDC9 (2), containing the pE194 replication functions and the *cbh* gene; used as unstable integration vector; Em^r	12
pGAF001	Integration vector; pGIP73 derivative containing an *Sma*I linker in the unique *Xba*I site of the *cbh* gene; Em^r	This work
pGAF002	pGAF001 derivative containing the *cbh* gene disrupted by the α-amylase gene fragment from pYYZ2112 in the same transcriptional orientation; Em^r	This work
pYYZ2110–pYYZ2117	pGK13 derivatives containing different 5′ parts of the *L. amylovorus* α-amylase gene; Amy^+ Em^r Cm^r	This work
Strains		
L. amylovorus NRRLB4540	Isolated from cattle waste corn fermentation; Amy^+	19
L. plantarum NCIB8826	Isolated from human saliva	National Collection of Industrial Bacteria
L. plantarum Lp80	Grass silage starter	26
L. plantarum LPYYZ2112	Lp80 strain containing the autoreplicative plasmid pYYZ2112; Amy^+ Em^r Cm^r	This work
L. plantarum NCYYZ2112	NCIB8826 strain containing the autoreplicative plasmid pYYZ2112; Amy^+ Em^r Cm^r	This work
L. plantarum LPGAFΔ5	Stable Amy^+ Lp80 strain in which the *cbh* gene is disrupted in the same transcriptional orientation by the pYYZ2112 α-amylase gene fragment	This work
L. plantarum NCGAFΔ1	Stable Amy^+ NCIB8826 strain constructed like LPGAFΔ5	This work
E. coli TG1	K-12 Δ(*lac-pro*) *supE thi hsdD5* F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15	25

secretion signals originating from within the *Lactobacillus* genus are likely to function properly within another species of the same genus. Furthermore, a recombinant strain containing a heterologous gene originating within the same genus as the host strain would be more acceptable to regulatory agencies controlling the release of recombinant organisms into the environment.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are described in Table 1. MRS (Oxoid) and LB (25) media were used for routine culturing of lactobacilli and *Escherichia coli*, respectively. Amylase activity was detected by incorporation of 0.2% starch in the media described above and subsequent visualization of starch degradation halos by staining with iodine vapors. Antibiotics (Boehringer) were used at the following final concentrations: chloramphenicol at 20 μg/ml and erythromycin at 200 μg/ml for *E. coli*, and chloramphenicol at 5 or 10 μg/ml and erythromycin at 5 μg/ml for *L. plantarum*.

DNA manipulations and transformation. Restriction, ligation, calf intestine alkaline phosphatase treatment, DNA fragment electroelution, DNA sequencing, isolation of chromosomal DNA from *L. plantarum* or plasmid DNA from *E. coli*, and Southern blotting were all performed as described by Sambrook et al. (25). DNA probes were labeled with digoxigenin (DIG)-dUTP by using the DIG labeling kit supplied by Boehringer Mannheim or with [^{35}S]dATP by using the random labeling kit supplied by Bethesda Research Laboratories and were hybridized to Southern blots as directed in the kits. Transformation of *E. coli* and *L. plantarum* with plasmid DNA was performed with a Bio-Rad Gene Pulser unit as described by the manufacturer and by Josson et al. (16), respectively.

Determination of amylase activity. Culture supernatants for the determination of amylase activity were prepared by centrifugation at 15,000 × *g* for 10 min at 4°C. Cell extracts were prepared by washing pellets twice in half the original culture volume of 0.2 M sodium phosphate buffer, pH 5.5, and resuspending the cells in 1/10 the original culture volume of 0.2 M sodium phosphate buffer, pH 5.5. The cell suspension was then placed on ice and subjected to sonication for 10 cycles of 30 s each at 65 W at 30-s intervals with a Labsonic 2000 ultrasonic homogenizer. The sonicated cell suspension was diluted and plated in order to determine the number of cells remaining intact. The lysed cells were centrifuged at 5,000 × *g* for 10 min at 4°C to remove cell debris, and the supernatant was stored at −20°C. Amylase activity was determined with the Phadebas system (Pharmacia).

Nucleotide sequence accession numbers. The EMBL and GenBank accession number for the *L. amylovorus* α-amylase gene fragment is X80271. The GenBank accession number for the *L. plantarum* 16S rRNA sequence is M58826 (30).

RESULTS

Cloning and sequence analysis of the *L. amylovorus* DNA fragments conferring an α-amylase-positive phenotype. Chromosomal DNA was isolated from *L. amylovorus* NRRLB4540 and partially digested with *Mbo*I. DNA fragments between 2 and 10 kb in size were isolated by electroelution and ligated to *Bam*HI-digested, alkaline phosphatase-treated pGK13 vector DNA. *E. coli* TG1 cells were transformed with the ligation mix, plated on LB agar containing chloramphenicol (20 μg/ml) and 0.2% soluble starch, and incubated at 37°C for 48 h. The plates were stained with iodine vapor, and of the 10,000 transformants, 8 different amylase-positive clones, designated pYYZ2110 to pYYZ2117, were identified. Southern blotting showed that all eight clones had the same 1.3-kb *Hpa*II fragment, originating from the *L. amylovorus* chromosome (data not shown).

The nucleotide sequence of the 5′ end of the *L. amylovorus* amylase gene was determined with plasmid pYYZ2112 in order to confirm the gene orientation and that the cloned region of DNA contained the sequences required for the efficient expression of the amylase gene and for secretion of

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                                    >> >> >< << <<
GCAAAAAAATTTTCGATTTTTATGAAAACGGTTGCAAAGAAGTTAGCAAAAATATATAAT      60
                                   -35              -10

TTCTTTTGAAATTGTTCACTTGGCCAAGCTGCAGTTTCAATATTTTAATAAAGGGGGCAG     120
                                                  SD

TAAAAAGTGAAAAAAAAGAAAAGTTTCTGGCTTGTTTCTTTTTTAGTTATAGTAGCTAGT     180
     M  K  K  K  K  S  F  W  L  V  S  F  L  V  I  V  A  S

GTTTTCTTTATATCTTTTGGATTTAGCAATCATTCTAAACAAGTTGCTCAAGCGGCTAGT     240
V  F  F  I  S  F  G  F  S  N  H  S  K  Q  V  A  Q  A  A  S

GATACGACATCAACTGATCACTCAAGCAATGATACAGCTGATTCTGTTAGCGACGGTGTT     300
D  T  T  S  T  D  H  S  S  N  D  T  A  D  S  V  S  D  G  V

ATTTTGCATGCATGGTGCTGGTCGTTCAACACGATTAAAAACAACTTGAAACAGATTCAT     360
I  L  H  A  W  C  W  S  F  N  T  I  K  N  N  L  K  Q  I  H

GACGCCGGCTACACAGCGGTTCAAACTTCACCTGTTAATGAAGTTAAAGTTGGAAATAGC     420
D  A  G  Y  T  A  V  Q  T  S  P  V  N  E  V  K  V  G  N  S

GGGTCTAAGTCATTAAATAACTGGTATTGGCTATATCAGCCAACTAAATATAGTATTGGT     480
G  S  K  S  L  N  N  W  Y  W  L  Y  Q  P  T  K  Y  S  I  G

AACTATTATTTAGGAACGGAAGCTGAATTTAAGTCAATGTGCGCTGCTGCTAAAGAATAT     540
N  Y  Y  L  G  T  E  A  E  F  K  S  M  C  A  A  A  K  E  Y

AATATCAGGATCATTGTCGATGCAACTCTGAATGATACAACAAGTGATTAT              591
N  I  R  I  I  V  D  A  T  L  N  D  T  T  S  D  Y
```

FIG. 1. Partial sequence of the *L. amylovorus* α-amylase gene (N-terminal end). The −35 and −10 boxes of the putative promoter are indicated, as is the ribosome-binding site (SD; bold underline). The presumed signal peptidase cleavage site is indicated by an arrow. Above the −35 box is shown the putative glucose catabolite repression operator, with its palindromic structure indicated by opposing arrowheads.

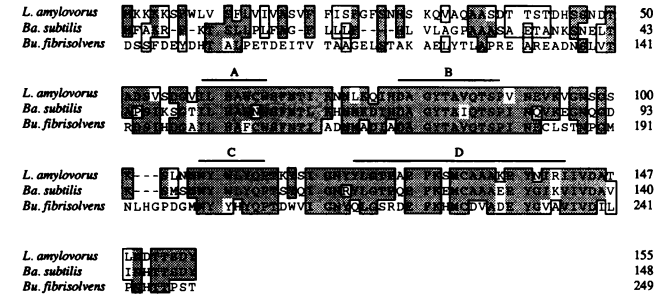

FIG. 2. Similarity between α-amylases from *L. amylovorus* (this work), *B. subtilis* (32), and *B. fibrisolvens* (24). Numbers to the right refer to amino acid positions starting at the N-terminal end of the preprotein. Similar amino acids are boxed, and identical residues are shaded. A, B, C, and D indicate regions of similarity conserved throughout the α-amylase family (24).

the corresponding protein. It can be seen (Fig. 1) that the α-amylase open reading frame starts with a GUG codon preceded at a canonical distance (9 nucleotides) by a putative ribosome-binding site (AAAGGGGG) complementary to the 3′ end of the *L. plantarum* 16S rRNA taken as a reference (UCUUUCCUCCAC; complementary bases underlined) (30). A potential promoter with the sequences TATAAT (−10 box) and TTGCAA (−35 box) is found further upstream, in agreement with the consensus of promoters from gram-positive bacteria (11) and other genes from lactobacilli (22). A putative operator for glucose repression similar to that found in *Bacillus subtilis* (29) is found to overlap the −35 box, as already observed for the xylose genes in *Lactobacillus pentosus* and *Lactobacillus brevis* (22). Translation of the open reading frame starts with a tripartite amino acid sequence typical of Sec-dependent signal peptides (28), made of a short positively charged region followed by a hydrophobic core preceding a signal peptidase cleavage site obeying the −3, −1 rule (28). Sequencing of the 3′ end of the insert revealed the absence of a translation stop in the open reading frame, indicating that only an active fragment of the *L. amylovorus* gene had been cloned. The sequenced N-terminal fragment of the *L. amylovorus* α-amylase was found to be quite close (Fig. 2) to those of the α-amylases from *B. subtilis* (32) and *Butyrovibrio fibrisolvens* (24).

Construction of amylolytic inoculant strains of *L. plantarum*. An amylolytic derivative of the inoculant strain *L. plantarum* Lp80 was constructed by transforming Lp80 with the autoreplicative plasmid pYYZ2112, yielding *L. plantarum* LPYYZ2112. However, as discussed previously, any potential inoculant strain must satisfy the regulations governing the release of recombinant organisms into the environment, which dictate that strains carrying heterologous genes stably integrated into the host chromosome and free of any residual vector sequences are more acceptable than their counterparts carrying autoreplicative plasmids. A strategy for the integration of the *L. amylovorus* amylase gene into the *L. plantarum* Lp80 chromosome by using plasmid pGIP73 was therefore devised. This plasmid contains both gram-negative and gram-positive origins of replication, originating from pJDC9 and pE194, respectively; a gene coding for erythromycin resistance; and the conjugated bile acid hydrolase gene (*cbh*) from *L. plantarum*, within which is situated a unique *Xba*I site (4).

Restriction analysis of pYYZ2112 revealed that it contained no sites facilitating the subcloning of the region coding for amylase activity into the *Xba*I site of pGIP73. An *Sma*I adaptor with the sequence 5′-CTAGCCCGGG-3′ was therefore introduced into the *Xba*I site of pGIP73. The resulting plasmid, pGAF001, was restricted with *Sma*I and ligated to a 3.2-kb *Eco*RV-*Stu*I fragment from pYYZ2112 which contained the sequences coding for amylase activity. The resulting construct, pGAF002, contained the regions coding for amylase activity from *L. amylovorus* positioned within the *cbh* gene. *L. plantarum* Lp80 was transformed with pGAF002 DNA, which was expected to direct integration of the DNA coding for amylase production at the Lp80 *cbh* chromosomal locus, as shown in Fig. 3A and B. Six amylase-positive, erythromycin-resistant transformants of *L. plantarum* Lp80 were cultured for 30 generations in MRS broth containing erythromycin (5 μg/ml) to allow the initial integration event to take place. The cells were then spread on MRS plates supplemented with 0.2% starch and erythromycin (5 μg/ml), and two amylase-positive colonies from each of the original six cultures were grown for 10 generations in MRS broth without selection. The cells were spread on MRS plates supplemented with 0.2% starch, and 8 of the 12 cultures were found to stably maintain amylase activity. It was presumed that the desired integration event had occurred in these eight cultures, which were then maintained for 30 generations in MRS broth without selection to allow excision of DNA originating from plasmid pGIP73 to occur. Two excision events were possible at this point, resulting in an erythromycin-sensitive and an amylase-positive or -negative phenotype. Cells with the desired phenotype (Amy^+ Em^s) were identified by growing them on MRS plates containing 0.2% starch and replica plating amylase-positive colonies on

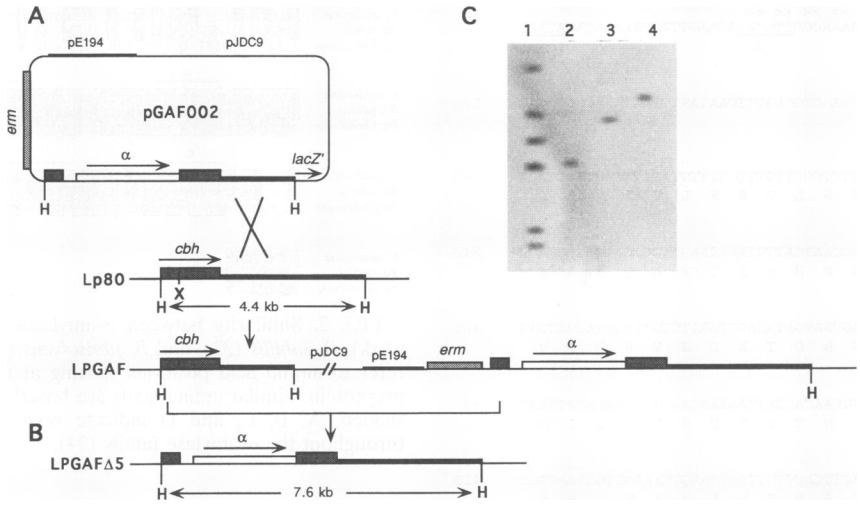

FIG. 3. Two-step stable chromosomal integration of the pYYZ2112 α-amylase gene fragment into *L. plantarum*. (A) Homologous recombination between the unstable autoreplicative plasmid pGAF002 and the Lp80 chromosome taking place in the larger 3′ moiety of the *cbh* gene to yield the LPGAF intermediate configuration at the *cbh* locus. (B) Intrachromosomal recombination between the tandemly repeated 5′ *cbh* fragments present at the site of integration in the LPGAF strain, to yield the LPGAFΔ5 strain. This transgenic strain therefore contains, in the *Xba*I restriction site of the *cbh* gene, a unique copy of the α-amylase gene fragment in the same transcriptional orientation as *cbh*. H, *Hin*dIII; X, *Xba*I; α, amylase insert; *erm*, erythromycin resistance marker. (C) Southern blot hybridization of the ^{35}S-labeled, *Sma*I-linearized pGAF001 to *Hin*dIII digests of chromosomal DNA from Lp80 (lane 2) and LPGAFΔ5 (lane 3). Lane 1, phage λ digested with *Hin*dIII; lane 4, *Sma*I-digested pGAF001.

MRS plates with and without erythromycin. Approximately 60% of the cells were amylase positive, of which 15% were erythromycin sensitive.

Integration of the *L. amylovorus* α-amylase gene at the desired location in the *L. plantarum* Lp80 chromosome in a single clone, designated *L. plantarum* LPGAFΔ5, was confirmed by Southern blotting. The *Hin*dIII fragment containing the *cbh* gene (4) displayed an increased size of 7.6 kb in the disrupted strain, as opposed to 4.4 kb in wild-type strain Lp80, confirming the insertion of the 3.2-kb amylase fragment without any additional rearrangement (Fig. 3C). The above procedures were repeated with the laboratory strain *L. plantarum* NCIB8826, yielding the recombinant strains *L. plantarum* NCYYZ2112 and *L. plantarum* NCGAFΔ1, in which the amylase coding regions from *L. amylovorus* were maintained on the autoreplicative plasmid pYYZ2112 and integrated into the host chromosome, respectively.

Characterization of *L. plantarum* LPGAFΔ5. The stability of *L. plantarum* LPGAFΔ5 was compared with that of *L. plantarum* LPYYZ2112, in which the α-amylase gene of *L. amylovorus* was maintained on an autoreplicative plasmid. Only 1% of *L. plantarum* LPYYZ2112 CFU but 100% of *L. plantarum* LPGAFΔ5 CFU maintained amylase activity after 50 generations of growth under nonselective conditions. Colony replication showed that the LPYYZ2112 amylase-negative derivatives had lost the vector markers, indicating that the vector bearing the α-amylase insert had indeed been lost by segregational instability. High instability of the intact pGK13 vector has already been observed (15).

Table 2 shows the quantity and percentage of total amylase secreted into the extracellular medium by *L. amylovorus* and by *L. plantarum* Lp80 and NCIB8826 carrying the *L. amylovorus* amylase gene fragment on an autoreplicative plasmid or integrated into the host chromosome after 24 h of growth in MRS containing 0.5% starch and 0.5% glucose as the fermentable carbohydrate source, plus chloramphenicol (10 μg/ml) in the case of autoreplicative plasmids. The recombinant strains of *L. plantarum* Lp80 and *L. plantarum* NCIB8826 carrying DNA coding for the *L. amylovorus* α-amylase integrated into the chromosome secreted 72 and 37% of the amylase secreted by their autoreplicative counterparts, respectively, on a per-CFU basis. *L. plantarum* Lp80 seemed to be a less suitable host for amylase production than *L. plantarum* NCIB8826, secreting 46% (autoreplicative) to 91% (integrant) of the amylase secreted by the corresponding strains of *L. plantarum* NCIB8826. A similar trend has already been observed for the production of levanase in the same pair of strains transformed with autoreplicative plasmids carrying a levanase gene (12). Only *L. plantarum* NCYYZ2112 secreted more amylase per CFU than *L. amylovorus*, while *L. plantarum* NCGAFΔ1 and *L. plantarum* LPGAFΔ5 secreted 54 and 49%, respectively, of the amylase secreted by *L. amylovorus*. All strains secreted 82 to 88% of the total amylase produced.

Growth characteristics and patterns of amylase production in the different strains. The growth characteristics and patterns of amylase production of *L. amylovorus*, *L. plantarum* Lp80, and *L. plantarum* LPGAFΔ5 grown in MRS with 0.5% glucose, 0.5% starch, or 0.5% starch plus 0.5% glucose as the ferment-

TABLE 2. Amylase secretion levels in *L. amylovorus* and recombinant *L. plantarum* strains

Strain	Extracellular amylase			% of total amylase secreted[a]
	U/liter	U/CFU	% of control	
L. amylovorus (control)	47.2	9.44×10^{-11}	100	88
L. plantarum NCYYZ2112	69.2	1.39×10^{-10}	147	82
L. plantarum NCGAFΔ1	25.8	5.14×10^{-11}	54	85
L. plantarum LPYYZ2112	35.6	6.45×10^{-11}	68	86
L. plantarum LPGAFΔ5	23.8	4.63×10^{-11}	49	82

[a] Total amylase secreted corresponds to the ratio between α-amylase secreted in the supernatant and α-amylase associated with the cell pellet after lysis.

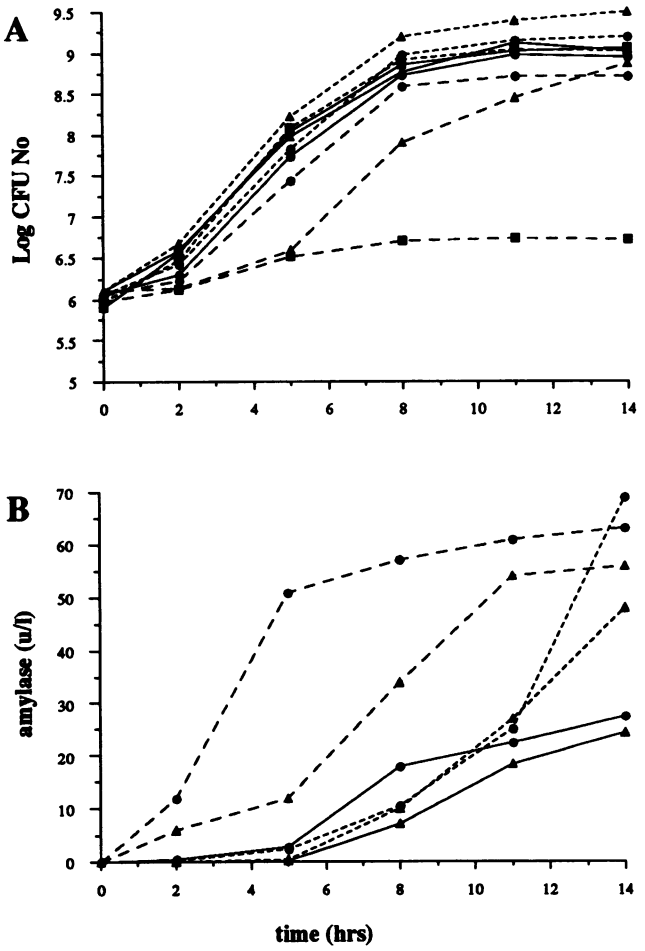

FIG. 4. Time course of growth (A) and amylase production (B) of *L. amylovorus* (circles), *L. plantarum* Lp80 (squares), and *L. plantarum* LPGAFΔ5 (triangles) on MRS. The sole carbon source was 0.5% glucose (——), 0.5% starch (— — —), or 0.5% glucose plus 0.5% starch (- - -).

able carbohydrate source are presented in Fig. 4. The amylolytic integrant *L. plantarum* LPGAFΔ5 displayed growth characteristics similar to those of the wild-type Lp80 strain when grown on glucose as the major carbohydrate source. However, the recombinant strain exhibited a marked competitive advantage over the wild-type strain when starch alone or a mixture of starch and glucose was used as the fermentable substrate. On starch alone, *L. plantarum* Lp80 failed to grow beyond a cell density of 5.54×10^6/ml, whereas *L. plantarum* LPGAFΔ5 cultures could reach a final cell density of 7.41×10^8/ml. The recombinant strain repeatedly displayed superior growth when a mixture of glucose and starch was used as the fermentable substrate. As shown in Fig. 4, after 14 h of growth, *L. plantarum* LPGAFΔ5 cultures displayed a cell density of 3.16×10^9/ml, compared with 1.13×10^9/ml for cultures of the wild-type strain.

Comparison between the LPGAFΔ5 α-amylase integrant and *L. amylovorus* again revealed superior growth of the former on glucose plus starch medium, whereas both strains behaved similarly on glucose medium. A comparatively long lag phase was seen in LPGAFΔ5 on starch alone, to be correlated with the low level of amylase production observed during the early stages of fermentation. Amylase production data show that the production of α-amylase is controlled by catabolite repression in both *L. amylovorus* and *L. plantarum* LPGAFΔ5, in that much less enzyme is produced in the presence of glucose (Fig. 4B).

DISCUSSION

In spite of secreting an amylase enzyme with temperature and pH profiles well suited to silage fermentation, *L. amylovorus* was found to be incapable of aiding the preservation of cereal silage under conditions in which *L. plantarum* Lp80 proved effective as an inoculant (7). This was consistent with the results of Petit and Flipot, who observed no effect of inoculation with a mixture of lactic acid bacteria including 5×10^9 *L. amylovorus* cells per g of forage on the pH or ammonia nitrogen content of cereal silage (21). We therefore decided to transfer the amylolytic activity of *L. amylovorus* to the competitive inoculant strain *L. plantarum* Lp80 by gene cloning techniques.

A region of DNA coding for the production of α-amylase was therefore cloned from the chromosome of *L. amylovorus*. Sequence data revealed that this region contained all the control elements necessary for efficient gene expression and protein secretion. The presence of these regions on the fragment of DNA to be used for integration into the *L. plantarum* chromosome was essential to obtain high levels of amylase secretion in the recombinant inoculant strain. However, the *L. amylovorus* α-amylase gene is 3′ end truncated. The complete gene codes for a larger protein whose size was estimated to 116 kDa (14) or 140 ± 10 kDa (1). Jore and DeParasis reported that the N-terminal part of the protein (45 kDa) still displayed full α-amylase activity and that the C-terminal part of the 116-kDa protein contains a second domain of unknown function (14). A similar case was recently reported for the α-amylase of *Butyrovibrio fibrisolvens* (24), whose N-terminal moiety displays a high similarity to that of *L. amylovorus*.

To construct an amylolytic strain of *L. plantarum* which would be effective as an inoculant and which would stably maintain amylase activity under nonselective conditions, the cloned fragment of the *L. amylovorus* α-amylase gene was integrated into the chromosome of the competitive inoculant strain *L. plantarum* Lp80 within the inessential conjugated bile hydrolase gene (*cbh*). For purposes of comparison, the *L. amylovorus* α-amylase gene was also integrated into the chromosome of the laboratory strain *L. plantarum* NCIB8826 and introduced into both strains on an autoreplicative vector. Characterization of the amylolytic integrant *L. plantarum* LPGAFΔ5 led to the following conclusions concerning its ensiling potential.

(i) When glucose was used as the sole fermentable carbohydrate source, *L. plantarum* LPGAFΔ5 was as competitive as the wild-type strain, *L. plantarum* Lp80. The competitiveness of the recombinant strain in the presence of glucose may be linked to the observed reduction of amylase production compared with that when glucose was absent, thereby ensuring that energy was not wasted in the large-scale production of amylase when the enzyme was not needed. A similar phenomenon was observed with *L. amylovorus*, suggesting that sequences regulating amylase production with regard to the carbohydrate environment were cloned and integrated into *L. plantarum* along with the *L. amylovorus* α-amylase gene. The presence near the promoter of the *L. amylovorus* α-amylase gene of an operator sequence similar to that responsible for glucose catabolite repression in *B. subtilis* (29) is consistent with this

view. The competitiveness of *L. plantarum* LPGAFΔ5, compared with the wild-type strain, also implies that integration within the *cbh* gene has no adverse effects on cell growth, confirming that the *cbh* gene is a suitable locus for chromosomal integration. Interestingly, a naturally occurring amylolytic *L. plantarum* strain isolated by Giraud et al. displayed a growth rate only 76% of that displayed by a typical *L. plantarum* strain (9), and given the importance of inoculant competitiveness, it is likely that *L. plantarum* LPGAFΔ5 would outperform this natural isolate in effecting preservation.

(ii) The integrant *L. plantarum* LPGAFΔ5 displayed a competitive advantage over *L. plantarum* Lp80 when grown on starch as the sole fermentable carbohydrate source. However, since all forage crops contain some glucose or fructose, the fermentation characteristics of *L. plantarum* LPGAFΔ5 on a mixture of starch and glucose are of more relevance to inoculation of silage. The use of MRS containing 0.5% glucose and 0.5% starch is representative of the situation frequently encountered in ensiling alfalfa, when available carbohydrates are insufficient to allow *L. plantarum* inoculants to produce enough lactic acid to ensure preservation while a reserve of starch remains unfermented. Under these conditions, *L. plantarum* LPGAFΔ5 still exhibits a gain in competitiveness, since the final cell density consistently reaches a higher CFU titer. These laboratory data suggest that *L. plantarum* LPGAFΔ5 would be capable of effecting preservation under such circumstances by utilizing starch to maintain its initial high rate of growth and lactic acid production.

The competitive advantage of *L. plantarum* LPGAFΔ5 over the wild-type *L. plantarum* Lp80 on starch-based media was due to a high level of amylase production by the integrant strain; after 24 h of growth on MRS containing 0.5% starch and 0.5% glucose, culture supernatants of *L. plantarum* LPGAFΔ5 contained 23.8 U of amylase per liter. This represents 49% of the amylase produced by *L. amylovorus* under similar conditions. By contrast, in the only comparable study, Scheirlinck et al. observed barely detectable levels of amylase activity when they integrated the α-amylase gene of *B. stearothermophilus* into the chromosome of *L. plantarum* Lp80 (26). Two factors may contribute to the superior levels of amylase production observed in this study. The promoter, ribosome-binding site, and signal sequence of the integrated α-amylase gene, though not originating from *L. plantarum*, still originate from within the *Lactobacillus* genus and would therefore be expected to be more compatible with the expression and secretion mechanisms of *L. plantarum* than those of sequences originating from *Bacillus* spp. Furthermore since the *L. amylovorus* α-amylase gene was integrated into the chromosomal *cbh* gene of *L. plantarum* Lp80 in the same orientation as the *cbh* gene, the *cbh* promoter may stimulate expression of the α-amylase gene along with the gene's own promoter.

While amylase expression levels in *L. plantarum* LPGAFΔ5 and NCGAFΔ1 are high compared with corresponding published values, they represent 72 and 37%, respectively, of the amylase secreted by the corresponding strains carrying autoreplicative plasmids. The difference may be due to a gene dosage effect; the integrant strains contain only a single copy of the amylase gene in the chromosome, while numerous copies of the autoreplicative plasmids may exist. *L. plantarum* Lp80 derivatives consistently produced less amylase than similar derivatives of *L. plantarum* NCIB8826, with levels ranging from 46% (autoreplicative) to 91% (integrant). This suggests that genetic background plays a role in determining amylase production levels and that it would be worthwhile testing amylase production levels in a range of competitive *L. plantarum* strains.

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