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Use of Homologous Expression-Secretion Signals and Vector-Free Stable Chromosomal Integration in Engineering of *Lactobacillus plantarum* for α-Amylase and Levanase Expression

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The genuine α-amylase gene from *Bacillus licheniformis* (*amyL*) is not expressed in *Lactobacillus plantarum*, but replacement of the *amyL* promoter by a strong *L. plantarum* promoter leads to efficient expression of the gene and secretion of more than 90% of the α-amylase into the culture supernatant. A series of *L. plantarum* genetic cassettes (transcription and translation with or without secretion) were cloned by translation fusion of random DNA fragments to the silent *amyL* coding frame in the pGIP212 probe vector (P. Hols, A. Baulard, D. Garmyn, B. Delplace, S. Hogan, and J. Delcour, Gene 118:21–30, 1992). Five different cassettes were sequenced and found to harbor genetic signals similar to those of other gram-positive bacteria. The functions of the cloned cassettes and the cassettes isolated previously from *Enterococcus faecalis* were compared in *E. faecalis* and *L. plantarum*, respectively. All signals were well recognized in *L. plantarum*, but cassettes isolated from *L. plantarum* led to a low level of amylase production in *E. faecalis*, suggesting that the *L. plantarum* signals are more species specific. Six transcriptional or translational fusions were constructed to express the *Bacillus subtilis* levanase gene (*sacC*) in *L. plantarum*. All of these constructions were capable of inducing levanase production and secretion in the culture supernatant, and, furthermore, *L. plantarum* strains harboring the most efficient fusions could grow in MRS medium containing inulin as the major carbon source. Finally, a two-step chromosomal integration procedure was used to achieve efficient stabilization of an amylase construction without any residual resistance marker or vector sequence.

Lactobacillus plantarum is known to play a significant role in silage (25) and in the gut microflora of mammals (37). This lactic acid bacterium has been used as an inoculum to stimulate lactic acid fermentation in grass silage (25), meat (1), and vegetables (11) and to enhance malolactic fermentation in wine (34). Furthermore, its generally recognized as safe status makes it potentially useful in the development of probiotics and oral or vaginal vaccines.

Most of the *L. plantarum* recombinant strains constructed to date have been constructed in order to improve silage additives. The total contents of fermentable sugars in many inoculated grass silages are too low to allow production of enough lactic acid to ensure preservation (25). This situation could be overcome by adding to the bacterial inoculum different hydrolases (cellulases, hemicellulases, pectinases, amylases, levanases, etc.) which could release free soluble sugars from carbohydrate polymers that are not accessible to lactic acid bacteria. The incorporation of such hydrolase activities in *L. plantarum* through genetic engineering could potentially improve the competitive position of this organism in silage and reduce inoculum cost.

Workers have developed a number of important tools for genetic manipulation of *L. plantarum*, including electroporation protocols (5, 18, 32) and cloning vectors (4, 18, 32, 33). A large number of heterologous genes from members of various gram-positive genera, such as the genera *Bacillus*, *Clostridium*, and *Butyrivibrio*, have been expressed in *L. plantarum* with some success (2, 17, 39, 40), and the high levels of segregational instability of many *L. plantarum* cloning vectors (33) have been circumvented by stably integrating heterologous genes into the chromosome (20, 35, 40).

However, a number of problems remain in developing genetically engineered *L. plantarum* strains for agroindustrial use. Previously reported levels of heterologous gene expression in *L. plantarum* are too low for practical applications. The integration systems that have been developed thus far are unsatisfactory with respect to complete stabilization of the foreign gene and to the food grade status of the engineered strains obtained. Only three *L. plantarum* chromosomal genes have been cloned and sequenced to date (3, 8, 10), and it is clear that isolation of a larger number of homologous expression and secretion signals will be necessary in order to achieve efficient heterologous expression and secretion.

In this paper, we describe the isolation and characterization of a range of expression and secretion signals from *L. plantarum* through the use of a probe vector approach, and we report the successful use of these signals for α-amylase and levanase production in *L. plantarum*. Furthermore, we describe a two-step integration procedure that allows stable integration into the *L. plantarum* chromosome of heterologous genes devoid of any resistance marker or vector sequence.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table

TABLE 1. Plasmids and bacterial strains

Plasmid(s) or strain	Genotype or relevant features	Origin or reference
Plasmids		
pNZ12	Shuttle vector for *E. coli* and *Lactococcus lactis*; Km^r, Cm^r	9
PGID023	Shuttle vector for *E. coli* and *L. plantarum*; derivative of pJDC9 containing the pE194 replication functions; used as an unstable integration vector; Em^r[a]	This study
pMC1	Shuttle vector for *E. coli* and *Bacillus subtilis* carrying the *sacC* gene from *Bacillus subtilis* in a 3.5-kb insert; Cm^r	23
pJC2	*E. coli* plasmid carrying the *sacC* gene in a 2.8-kb insert; Cm^r	23
pGIP51	*E. coli* plasmid; derivative of pBluescript SK^- (Stratagene) carrying the *sacC* translation unit in a 2.2-kb insert; Ap^r	This study
pH3	*E. coli* plasmid carrying the *cbh* gene from *L. plantarum* in an 8.0-kb insert; Em^r	8
pGIP31	Shuttle plasmid for *E. coli* and *L. plantarum*; derivative of pNZ12 carrying the *amyL* gene from *Bacillus licheniformis*; Km^r Cm^r	This study
pGIP311	pGIP31 derivative carrying the transcription terminators from pHP45Ω	This study
pGIP331	pGIP311 derivative from which the *amyL* promoter is deleted; used as a promoter probe vector; Km^r Cm^r Sm^r (*E. coli*)	13
pGIP332	pGIP331 derivative from which the transcription terminators are removed; Km^r Cm^r	13
pGIP212	Shuttle plasmid for *E. coli* for *L. plantarum*; used as expression-secretion probe vector; Em^r Cm^r Sm^r (*E. coli*)	13
pGIT008	pGIP331 derivative carrying a strong *L. plantarum* promoter in front of the deleted *amyL* gene; Km^r Cm^r Sm^r (*E. coli*)	This study
pGIP312.4	pGIP312 derivative containing expression-secretion signals from *Enterococcus faecalis*[b]	13
pGIP212.1, pGIP212.2, pGIP212.4, pGIP212.5	pGIP212 derivatives containing chromosomal inserts from *Enterococcus faecalis* OG1X	13
pGIP212.7, pGIP212.8, pGIP212.9, pGIP212.10, pGIP212.11, pGIP212.12, pGIP212.13	pGIP1212 derivatives containing chromosomal inserts from *L. plantarum* NCIB8826	This study
pGIP212.16, pGIP212.17, pGIP212.18, pGIP212.19	Derivatives of pGIP212.1, pGIP212.9, pGIP1212.10, and pGIP1212.13 containing *sacC* transcriptional fusions	This study
pGIP212.20, pGIP212.21	Derivatives of pGIP1212.12 and pGIP1212.1, respectively, containing *sacC* translational fusions	This study
pGIP741	Integration plasmid; pGID023 derivative containing the *cbh* gene disrupted by the α-amylase construction from pGIP212.1 in the same transcriptional orientation; Em^r	This study
pGIP742	Similar to pGIP741, with the α-amylase construction in the opposite orientation	This study
Strains		
L. plantarum NCIB8826	Isolated from human saliva	NCIB[c]
L. plantarum Lp80	Grass silage starter	18
L. plantarum Lp811	Stable Amy^+ Lp80 strain in which the *cbh* gene is disrupted in the same transcriptional orientation by the pGIP212.1 α-amylase construction	This study
L. plantarum Lp812	Similar to Lp811, with the α-amylase fusion in the opposite orientation	This study
Enterococcus faecalis OG1X	*str gel*	15
E. coli TG1	K-12 Δ(*lac-pro*) *supE thi hsdD5* F′ *traD36 proA*$^+$ *B*$^+$ *lacI*^{q} *lacZ*ΔM15	36

[a] See reference 6.
[b] See reference 13.
[c] NCIB, National Collection of Industrial Bacteria, Terry Research Station, Aberdeen, Scotland.

1. MRS medium (Difco) and THB medium (Oxoid) were routinely used for the propagation of *L. plantarum* and *Enterococcus faecalis*, respectively. THB medium was also used for *L. plantarum* growth when a low level of glucose was required. A mixture of THB medium (12 g/liter) and MRS medium (3 g/liter) designated PHB medium was used to screen amylolytic *L. plantarum* transformants. *Escherichia coli* was routinely grown in Luria-Bertani medium (36). The saccharolytic activity associated with the *sacC* gene was detected on MacConkey agar (Difco) plates supplemented with 2% sucrose (23), and conjugated bile acid hydrolase activity was detected by using the $CaCl_2$-taurodeoxycholic acid-based medium described by Christiaens et al. (8). Amylase activity was detected by incorporating 0.2% starch in the media described above and subsequently visualizing starch degradation halos by staining with iodine vapors. Antibiotics (Boehringer) were used at the following final concentrations: 10 μg of tetracycline per ml, 100 μg of ampicillin per ml, 20 μg of chloramphenicol per ml, 200 μg of erythromycin per ml, 25 μg of streptomycin per ml, and 10 μg of chloramphenicol plus 12.5 μg of streptomycin per ml for *E. coli*; and 5 or 10 μg of chloramphenicol per ml and 10 μg of erythromycin per ml for *L. plantarum* and *Enterococcus faecalis*.

DNA manipulations and transformation. Plasmid DNA from *L. plantarum* was prepared as described by Posno et al. (32). Chromosomal DNA from this species was extracted by using a modification of the same method in which the NaCl precipitation step was omitted. Electroporation of *L. plantarum* NCIB8826 and Lp80 was performed as described by Josson et al. (18).

The following techniques were performed as described previously (13, 14): *E. coli* plasmid preparation and transfor-

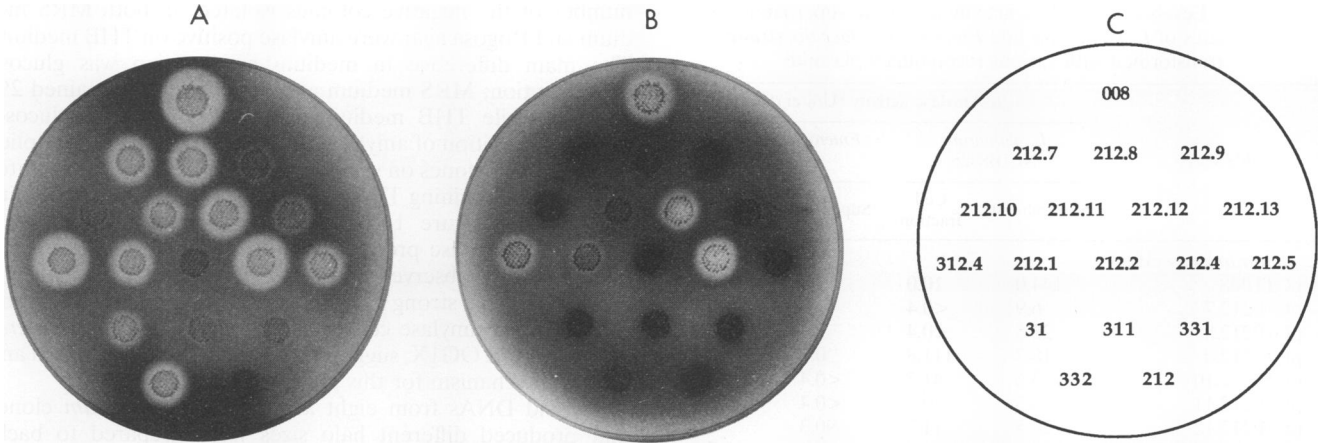

FIG. 1. Iodine plate test for α-amylase activities of *L. plantarum* NCIB8826 transformed with various plasmids. (C) Serial numbers of plasmids harbored by colonies. (A) THB medium supplemented with 0.2% soluble starch. (B) THB medium supplemented with 0.2% soluble starch and 2% glucose. Halos of starch degradation were visualized after 16 h of growth by staining the plates with iodine vapors.

mation; restriction; ligation; filling in of cohesive ends; agarose gel electrophoresis; PCR amplification; Southern blotting; and DNA sequencing.

Construction of the integration plasmids. Two integration plasmids, pGIP741 and pGIP742, were constructed as follows. The polylinker region between *Sma*I and *Sal*I was deleted in shuttle vector pGID023, which contains the pE194 replication origin, to yield pGIP72. This deletion eliminated a unique *Xba*I site from the polylinker. The 2.7-kb *Hin*dIII fragment from plasmid pH3 carrying the *cbh* gene (deleted of its promoter) was inserted into the *Hin*dIII site of pGIP72 to yield pGIP73. The amylase construction from pGIP212.1 was removed as a 2.0-kb *Xba*I fragment and was inserted into the unique *Xba*I site of pGIP73. Insertion was obtained in the two orientations corresponding to the pro- and antitranscriptional orientations of the *cbh* gene, and the two final constructions were designated pGIP741 and pGIP742, respectively.

Cell fractionation and enzymatic assays. An overnight culture of *L. plantarum* in MRS medium or of *Enterococcus faecalis* in THB medium was used to inoculate THB medium at a dilution of 1/100. The cultures used for amylase assays were grown for 16 h at 37°C, while the cultures used for levanase assays were grown for 24 h at 37°C. The cells were removed by centrifugation for 10 min at 4,500 × *g*, and the supernatants were stored at −20°C. The harvested cells (2×10^7 CFU/ml) were frozen overnight, resuspended to one-tenth of the original volume in distilled water, and incubated at 37°C for 90 min in the presence of 1 mg of lysozyme (Boehringer) per ml. Complete lysis was achieved by using glass beads (0.5 volume) and four 30-s treatments in a Braun homogenizer.

α-Amylase activity was assayed by using a Phedebas kit (Pharmacia). The test was performed as recommended by the manufacturer except for the following modifications: sample sizes of 100 or 1,000 μl were used, and the enzymatic mixture was incubated for 2 h at 55°C. The levels of amylase activity (in units per liter) were calculated by using the table provided with the kit. α-Amylase activity in sodium dodecyl sulfate-polyacrylamide gel electrophoresis starch gels (zymograms) was detected as previously described (13).

The levanase assay was based on a determination of the amount of fructose released from inulin degradation. The assay was performed at 37°C in 0.2 M potassium phosphate buffer (pH 5.5) containing 0.2 M potassium acetate, and 1% inulin was used as the substrate. The release of D-fructose was measured by using a D-glucose–D-fructose enzymatic test (Boehringer), and 1 U of levanase activity corresponded to the release of 1 μmol of fructose in 1 min.

Nucleotide sequence accession numbers. The EMBL and GenBank accession numbers are as follows: pGIP212.7, X71999; pGIP212.9, X73296; pGIP212.10, X72001; pGIP212.11, X72002; and pGIP212.12, X72003. The GenBank accession number for the *L. plantarum* 16 S rRNA sequence is M58826 (52).

RESULTS

Expression of the *Bacillus licheniformis* α-amylase gene (*amyL*) in *L. plantarum*. The ability of *L. plantarum* to express the *Bacillus licheniformis amyL* gene and to secrete amylase into the culture medium was tested by inserting the complete *amyL* transcription unit (a 2.5-kb insert) (31) into the unique *Sal*I restriction site of pNZ12. The resulting plasmid, pGIP31, was transferred by electroporation to *L. plantarum* NCIB8826, and small halos of starch degradation were observed around the transformants on starch-containing THB medium plates (Fig. 1).

The small halo sizes suggested that the levels of *amyL* expression were very low, possibly as a result of a deficiency in expression or secretion. To test this hypothesis, various genetic constructions were tested. We constructed plasmid pGIP311, which contains a strong transcriptional terminator in front of the complete *amyL* gene, and transfer of this plasmid to *L. plantarum* NCIB8826 resulted in a virtually complete loss of *amyL* expression (Fig. 1). *L. plantarum* NCIB8826 transformed with pGIP331, in which the *amyL* gene lacks its promoter and is protected by a terminator, failed to express amylase (Fig. 1). However, cells transformed with pGIP332, a pGIP331 derivative in which the terminator is absent (Fig. 1), were amylolytic, indicating that the *amyL* gene was expressed not from its own promoter but from promoters carried by the plasmid. Various strong promoters were isolated from the *L. plantarum* NCIB8826 chromosome by using the pGIP331 promoter

TABLE 2. Levels of α-amylase activity in culture supernatants and cell lysates of *L. plantarum* and *Enterococcus faecalis* strains transformed with various recombinant plasmids

Plasmid	α-Amylase activity (U/liter)[a]			
	L. plantarum NCIB8826		*Enterococcus faecalis* OG1X	
	Supernatant	Cell fraction	Supernatant	Cell fraction
L. plantarum cassettes				
pGIT008	144.0	10.0	58.4	0.4
pGIP212.7	6.9	<0.4	2.6	<0.4
pGIP212.8	22.5	<0.4	1.2	<0.4
pGIP212.9	10.7	111.8	0.8	2.6
pGIP212.10	3.6	41.7	<0.4	1.2
pGIP212.11	6.3	<0.4	<0.4	0.6
pGIP212.12	27.5	11.3	90.3	1.5
pGIP212.13	16.3	217.6	0.6	3.4
Enterococcus faecalis cassettes				
pGIP312.4	92.3	9.2	99.5	12.9
pGIP212.1	42.3	5.0	7.9	<0.4
pGIP212.2	3.5	104.5	3.5	7.8
pGIP212.4	92.5	11.6	75.3	2.8
pGIP212.5	12.4	2.1	10.6	0.6

[a] The minimum detectable level of activity in the assay was 0.4 U/liter.

probe-vector (data not shown). With one of the recombinant plasmids, designated pGIT008, in which the nonfunctional *amyL* promoter was replaced by a 2.0-kb fragment, we observed a high level of amylase production in *L. plantarum*, and more than 90% of the amylase activity was found in the culture supernatant (Table 2). Furthermore, the electrophoretic mobility of the α-amylase band on zymograms prepared from the culture supernatant of NCIB8826(pGIT008) was similar to the electrophoretic mobility of the mature α-amylase band originating from *Bacillus licheniformis* (data not shown). We therefore concluded that the *amyL* gene was a useful reporter of gene expression and protein secretion in *L. plantarum*.

Two-step screening for *L. plantarum* expression-secretion cassettes. The pGIP212 probe vector contains an *amyL* gene deleted of its own signals governing expression (promoter and ribosome binding site [RBS]) and secretion (signal peptide) (*'amyL*) and preceded by a strong terminator. *L. plantarum* NCIB8826 containing this plasmid was completely amylase negative (Fig. 1), and amylase production could be restored only by cloning expression-secretion signals in front of the *'amyL* gene.

To determine suitable screening conditions, a small-scale ligation mixture containing *Mbo*I-digested chromosomal DNA from *L. plantarum* NCIB8826 and *Bam*HI-digested pGIP212 was introduced into *E. coli* TG1 by electroporation, and 30 amylase-positive colonies were obtained on starch-containing Luria-Bertani agar plates supplemented with chloramphenicol and streptomycin. Plasmid DNAs from these pooled clones were introduced into *L. plantarum* NCIB8826 as described in Materials and Methods. The transformation mixture was spread onto MRS medium or Rogosa agar (Difco) plates supplemented with starch and chloramphenicol, but no Amy^+ colonies were obtained. This suggested that MRS medium and Rogosa agar were not suitable for screening purposes. The transformants were replica plated onto THB medium supplemented with starch and chloramphenicol (5 μg/ml), and a large number of the negative colonies isolated on both MRS medium and Rogosa agar were amylase positive on THB medium. The main difference in medium composition was glucose concentration; MRS medium and Rogosa agar contained 2% glucose, while THB medium contained only 0.2% glucose. Glucose inhibition of amylase production was tested by replica plating Amy^+ clones on starch-containing THB medium plates and starch-containing THB medium plates supplemented with 2% glucose. Figure 1 clearly shows the negative effect of glucose on amylase production in *L. plantarum*. This glucose effect was also observed with the original *amyL* gene and its derivative with a strong *L. plantarum* promoter (pGIT008) and with different amylase constructions originating from *Enterococcus faecalis* OG1X, suggesting that there was a common and general mechanism for this glucose effect.

Plasmid DNAs from eight amylolytic *L. plantarum* clones that produced different halo sizes were prepared to back-transform *E. coli* TG1. A restriction analysis revealed that the recombinant plasmids contained inserts ranging in size from 0.3 to 1.2 kb. Four clones containing 0.7-, 1.2-, 0.5-, and 0.3-kb inserts were designated pGIP212.7, pGIP212.8, pGIP212.9, and pGIP212.10, respectively, and were used for further analysis. The production of extracellular amylase and the production of intracellular amylase were compared in the corresponding *L. plantarum* recombinant clones (Table 2). Plasmids pGIP212.7 and pGIP212.8 were capable of directing secretion of more than 90% of the amylase produced, while in *L. plantarum* clones containing pGIP212.9 and pGIP212.10 (Table 2) amylase production was almost exclusively intracellular. The results of these fractionation experiments correlated with the halo sizes produced by the corresponding clones.

Direct screening for expression-secretion cassettes in *L. plantarum*. A large-scale ligation experiment was performed by using the conditions described above, and the ligation mixture was directly introduced into *L. plantarum* NCIB8826. The transformation mixture was spread onto starch-containing MRS medium plates supplemented with chloramphenicol (10 μg/ml), and 2×10^4 independent colonies were recovered from the MRS medium plates and spread onto starch-containing PHB medium supplemented with chloramphenicol (5 μg/ml) to screen for amylase production. Of the 10^5 colonies obtained on PHB medium, 41 were amylolytic, and 14 of these were retained for further study on the basis of halo size and glucose response. Plasmid DNAs were prepared from these clones and were transferred into *E. coli* TG1. Difficulties were encountered in the transfer of some recombinant plasmids; either back-transformation was associated with a modification of colony morphology, or back-transformants were not obtained at all, suggesting that amylase production directed by these constructions was deleterious to *Escherichia coli*, as observed previously (47). The problem was partially solved by adding 10 mM $MgSO_4$ to Luria-Bertani medium, as suggested previously (47). The insert sizes and partial restriction maps of the cloned inserts revealed three different cassettes. The corresponding plasmids were designated pGIP212.11, pGIP212.12, and pGIP212.13 and contained inserts that were 2.2, 0.9, and 0.9 kb long, respectively. The small proportion of distinct clones (3 of 14 clones) resulted from the intermediate amplification of the library on PHB medium plates. Cell fractionation experiments performed with the corresponding *L. plantarum* clones (Table 2) revealed that the pGIP212.11 cassette directed secretion of more than 90% of the amylase produced. Only 60% secretion was obtained with pGIP212.12, and no significant extracellular production was observed with pGIP212.13. This intracellular production result was comparable to the result obtained previously with the pGIP212.9 and

pGIP212.10 constructions. Restriction analysis revealed that pGIP212.13 differed from pGIP212.9 only by containing an additional 0.4-kb fragment at the 5′ end of the insert. This 5′ DNA fragment probably carried an additional promoter or *cis*-acting sequence that enhanced transcription since its presence was correlated with a twofold increase in intracellular amylase production, as observed when NCIB8826(pGIP212.13) was compared with NCIB8826(pGIP212.9) (Table 2).

Nucleotide sequences analysis. Most of the cassettes were sequenced from their 3′ ends up to at least 120 bp upstream of the translation start (Fig. 2). Sequencing was particularly difficult since high levels of segregational instability of plasmids bearing amylase constructions were observed in *E. coli*. This was also true when the expression signals were separately cloned into sequencing vectors (phagemids), where they seemed to perturb plasmid replication. Thus, the sequence of the pGIP212.8 cassette could not be completed despite repeated attempts. The sequence at the 3′ end of the pGIP212.13 cassette was identical to the sequence at the 3′ end of pGIP212.9, as suggested by the results of the restriction analysis (see above).

A sequence analysis revealed that there were two types of *'amyL* fusions which correlated with the fractionation results shown in Table 2. In the pGIP212.9 and pGIP212.10 cassettes, the translational start was close to the fusion point. In contrast, the peptides encoded by the pGIP212.7, pGIP212.11, and pGIP212.12 cassettes were 44, 82, and 56 amino acids long, respectively, and had at their N termini the typical tripartite structure (n, h, and c regions) of a signal peptide (43). The n regions upstream of the hydrophobic core were 7, 17, and 8 amino acids long, and their charges were +2, +3, and +3, respectively. The hydrophobic cores (h region) were rather long (20, 18, and 15 amino acids, respectively), but the lengths were within values common in gram-positive bacteria (43). Signal peptidase cleavage sites conforming to the −3, −1 rule (51) were identified in all of the peptides. In the pGIP212.11 and pGIP212.12 transformants, cleavage actually occurred at one of these sites since the enzymes from culture supernatants were significantly larger than the mature α-amylase of *Bacillus licheniformis*. In contrast, the supernatant enzyme from pGIP212.7 transformants was the same size as mature α-amylase, indicating that cleavage took place at the proper α-amylase signal cleavage site or that postsecretion processing by extracellular proteases occurred (data not shown). We noted that in the pGIP212.12 signal peptide the SIA/C cleavage site, which is the best candidate for signal peptidase I as determined by the weight matrix method (50), could also be a cleavage site for signal peptidase II, which is responsible for the processing of secreted lipoproteins (43, 51).

Putative Shine-Dalgarno (SD) sequences having high negative ΔG values ranging between 12.2 and 21.6 kcal/mol (48) were 6 to 11 nucleotides upstream of an ATG or GTG start codon, which is consistent with translation signals found in other gram-positive bacteria (Fig. 3) (26). Putative additional interactions between the small ribosomal subunit and the translation start site were suggested by the presence of sequence elements close to the SD box and complementary to the 5′ end of 16S rRNA from *L. plantarum* (52). The corresponding rRNA-mRNA hybrids had negative ΔG values ranging between, 4.8 and, 13.8 kcal/mol (average, 8.0 kcal/mol) (Fig. 3).

Expression signals from *L. plantarum* are poorly recognized in *Enterococcus faecalis*. Previously (13), expression-secretion cassettes from *Enterococcus faecalis* were isolated and analyzed. The corresponding plasmids (pGIP312.4, pGIP212.1, pGIP212.2, pGIP212.4, and pGIP212.5) were transferred to *L. plantarum* NCIB8826 to determine their ability to produce amylase in this host. pGIP312.4 and pGIP212.4 directed amylase production to about the same level in both species, whereas pGIP212.1 and pGIP212.2 were expressed much more efficiently in *L. plantarum* than in *Enterococcus faecalis* (Table 2). In contrast, when most of the plasmids containing the expression or expression-secretion cassettes from *L. plantarum* were transferred to *Enterococcus faecalis* OG1X, there was a drastic reduction in amylase production. Efficient secretion of amylase in *Enterococcus faecalis* transformants containing *L. plantarum* cassettes capable of directing expression and secretion in their original hosts, as well as reduced expression from cassettes with only expression signals, suggested that the blockage was not at the secretion level but most probably at the transcriptional level and/or the translational level.

Levanase production in *L. plantarum* through transcriptional fusion. The *sacC* levanase gene from *Bacillus subtilis* was chosen to test the ability of the cloned cassettes to drive expression and/or secretion of another exoenzyme not present in *L. plantarum*. The *sacC* gene is the last gene of an operon whose promoter is located 2.7 kb upstream (24). Transformation with plasmid pMC1, which contains a 3.5-kb insert carrying the *sacC* gene without its promoter, did not confer any intracellular or extracellular levanase activity on *L. plantarum* NCIB8826 (Table 3). Therefore, promoters from the pMC1 vector framework were not capable of transcriptional activation of the *sacC* gene, unlike what was observed with the *amyL* gene and pNZ12.

The first stage in restoring *sacC* expression involved the construction of transcriptional fusions between different promoters encoded in pGIP212.1, pGIP212.9, pGIP212.10, and pGIP212.13 cassettes and the promoterless *sacC* gene. The *'amyL* gene was excised from the corresponding plasmids by *Pst*I-*Sma*I restriction and was replaced by a 2.2-kb *Pst*I-*Sca*I fragment from plasmid pJC2 containing the *sacC* translation unit. The resulting plasmids, pGIP212.16, pGIP212.17, pGIP212.18, and pGIP212.19, had a common fusion point, as shown in Fig. 4A. Two successive open reading frames (ORFs) could be translated. The first ORF was translated from the RBS included in the cassette and ended 21 nucleotides before the proper *sacC* RBS, where translation could genuinely be reinitiated. The four transcriptional fusions directed extracellular levanase production in *L. plantarum* NCIB8826 with a high level of secretion efficiency (more than 90%) (Table 3). Therefore, the proper *sacC* RBS and signal peptide are well recognized in *L. plantarum*. An intriguing result was the finding that levanase production was twofold greater in NCIB8826 (pGIP212.17) than in NCIB8826(pGIP212.19) (Table 3), since the production ratio was reversed in the recombinant strains containing the corresponding α-amylase constructions (Table 2). These two plasmids were also transferred to strain Lp80, and in this case levanase production was reduced equally in the two corresponding recombinant strains (Table 3).

Levanase production in *L. plantarum* through translational fusion. The potential use of the *L. plantarum* or *Enterococcus faecalis* signal peptides to direct secretion of an exoenzyme other than α-amylase was investigated. We constructed two translational fusions between the pGIP212.12 and pGIP212.1 expression-secretion cassettes and a *'sacC* gene deleted of its own expression and secretion signals. PCR amplification was performed with pGIP51 by using a 5′ primer covering the original peptide signal cleavage site (Fig. 4A) and a 3′ primer complementary to the vector polylinker (5′-GGAATTAAC CCTCACTAAAG-3′). The PCR fragment was restricted with *Pst*I and subcloned in plasmid pBluescript KS$^+$. The *Pst*I-*Sca*I *'sacC* fragment from this intermediate plasmid was used to

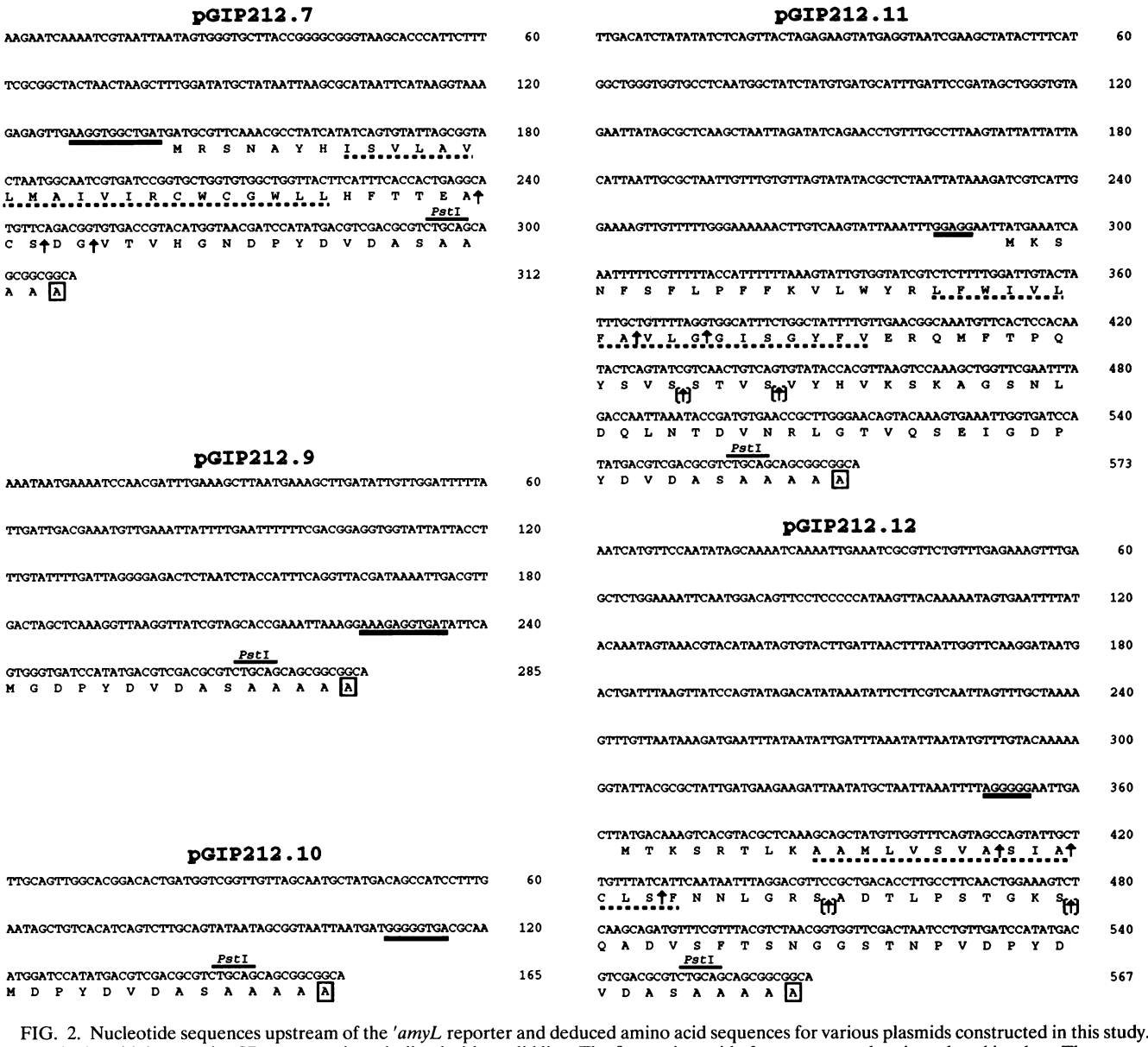

FIG. 2. Nucleotide sequences upstream of the *'amyL* reporter and deduced amino acid sequences for various plasmids constructed in this study. For each plasmid the putative SD sequence is underlined with a solid line. The first amino acid of mature α-amylase is enclosed in a box. The arrows indicate the positions of potential signal peptidase cleavage sites as determined by the weight-matrix method (50). The arrows in brackets indicate putative cleavage sites predicted by the −3, −1 rule (51) and not detected by the weight-matrix method. The search for putative cleavage sites was limited to the first 60 amino acids. The hydrophobic core of the signal peptide is underlined with a dotted line.

replace the corresponding *Pst*I-*Sma*I *'amyL* fragment in plasmids pGIP212.12 and pGIP212.1 to yield pGIP212.20 and pGIP212.21, respectively. The resulting translational fusion is shown in Fig. 4B. Both of the NCIB8826 recombinants harboring these constructions were capable of secreting levanase (Table 3). However, the efficiency of levanase secretion in NCIB8826(pGIP212.21) (83%) was lower than the efficiency of amylase secretion in the corresponding strain, NCIB8826 (pGIP212.1), (93%), emphasizing the importance of the downstream sequence for the recognition of the signal peptide cleavage site.

Growth of levanase transformants on inulin. The phenotypic significance of levanase production in the best *L. plantarum* recombinant strains was evaluated by testing the ability of these strains to use inulin as a carbon source. The following three different media were used: MRS medium without glucose and MRS medium supplemented with either glucose or inulin. Growth (Fig. 5) and pH values (data not shown) were similar in the control and recombinant strains in glucose-containing MRS medium and MRS medium without glucose. In contrast, a significant increase in growth was observed with both the NCIB8826 and Lp80 recombinant strains in MRS medium supplemented with inulin (Fig. 5). After 70 h of culture the pH values of the NCIB8826 and Lp80 recombinant

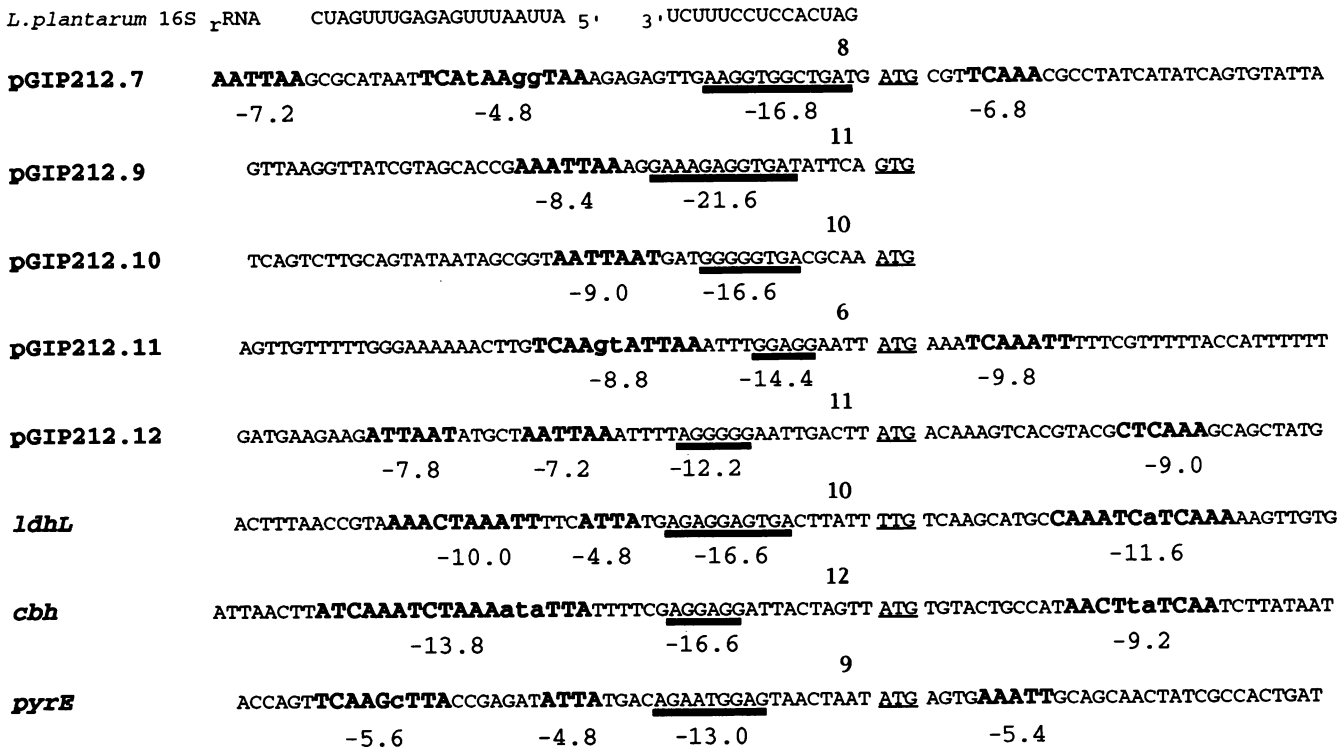

FIG. 3. SD sequences and their upstream regions found in various expression cassettes isolated from the *L. plantarum* genome and the corresponding nucleotide regions for *L. plantarum* chromosomal genes *ldhL* (10), *cbh* (8), and *pyrE* (3). For each plasmid or gene the SD sequence (thick line) and the start codon (thin line) are underlined. The putative boxes of hybridization with the 5′ end of *L. plantarum* 16S rRNA are indicated by boldface type (unpaired bases are in lowercase letters). The calculated ΔG values (in kilocalories per mole) (48) for the different putative duplexes with the 16S rRNA are indicated below the sequences. The distance between the SD sequence and the start codon is indicated above each sequence (the reference nucleotide was the G after the A or its equivalent in the center of the interaction). The sequences of the 5′ and 3′ termini of the *L. plantarum* 16S rRNA are shown at the top.

strains decreased from an initial value of 7.5 to 4.5 and 4.0, respectively, in glucose-containing MRS medium and to 5.0 and 4.5, respectively, in inulin-containing MRS medium, whereas the pH values remained constant in MRS medium without glucose. These in vivo data showed that production and secretion of levanase in two different *L. plantarum* strains, a culture collection strain (NCIB8826) and a commercial silage additive (Lp80), allowed the strains to efficiently use inulin as a carbon source for growth and acid production.

TABLE 3. Levels of levanase activity in the culture supernatants and cell lysates of *L. plantarum* strains transformed with various recombinant plasmids

Strain	Levanase activity (U/liter)[a]	
	Supernatant	Cell fraction
NCIB8826	<2.0	<2.0
NCIB8826(pMC1)	<2.0	<2.0
NCIB8826(pGIP212.16)	38.0	<2.0
NCIB8826(pGIP212.17)	40.0	<2.0
NCIB8826(pGIP212.18)	13.0	<2.0
NCIB8826(pGIP212.19)	23.6	<2.0
NCIB8826(pGIP212.20)	7.2	<2.0
NCIB8826(pGIP212.21)	23.7	4.0
Lp80	<2.0	<2.0
Lp80(pGIP212.17)	10.8	<2.0
Lp80(pGIP212.19)	11.5	<2.0

[a] The minimum detectable level of activity in the assay was 2.0 U/liter.

Stable and vector-free integration of an α-amylase construction into the chromosome of *L. plantarum* Lp80. The *cbh* gene encoding the conjugated bile acid hydrolase isolated from *L. plantarum* Lp80 was chosen as a target for stable integration in order to evaluate the possible gene dosage effect encountered in the propagation of strains containing autoreplicative plasmids. This gene has been disrupted previously (7, 8) without any detrimental effect on the growth of *L. plantarum*.

We developed a strategy in which we used an unstable autoreplicative vector in a two-step procedure (integration followed by excision). The two integration plasmids, pGIP741 and pGIP742, were introduced into *L. plantarum* Lp80 by electroporation, and both recombinant strains had an amylolytic phenotype. Six individual colonies from Lp80(pGIP741) and Lp80(pGIP742) cultures were chosen and grown in liquid MRS broth for 30 generations in the presence of erythromycin. Dilutions of the different cultures were plated on PHB medium containing erythromycin and starch, and 12 amylolytic colonies corresponding to individual cultures were grown for 10 additional generations in MRS medium without erythromycin to test the stability of the *erm* marker. Dilutions of the corresponding cultures were spread onto MRS medium plates, and 100 colonies of each dilution were toothpick replicated onto

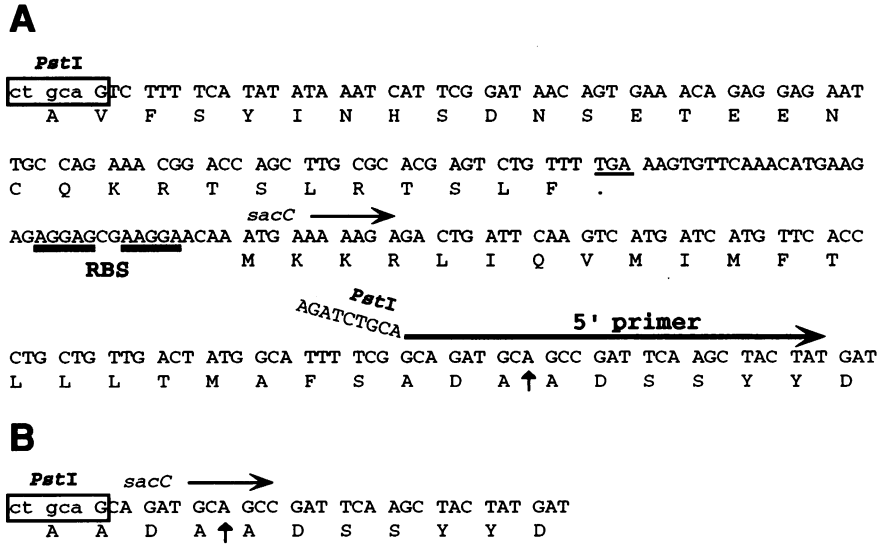

FIG. 4. Nucleotide sequence at the 5′ end of the *PstI-ScaI* fragment bearing the *sacC* gene in various levanase constructions. (A) Transcriptional fusions in plasmids pGIP212.16, pGIP212.17, pGIP212.18, and pGIP21219. The *PstI* restriction site (boxed) corresponds to the fusion point between the upstream expression or expression-secretion cassette and the *sacC* DNA fragment. An ORF beginning upstream in the cassette extends into the *sacC* DNA fragment through the *PstI* site and stops 21 nucleotides (underlined TGA) upstream of the proper *sacC* RBS (underlined with thick lines). The small vertical arrow indicates the position of the signal peptidase cleavage site of the levanase precursor as predicted by the weight-matrix method (50). The position of the PCR 5′ primer containing the floating *PstI* site used in the construction of the translational fusions (see below) is indicated. (B) Translational fusions in plasmids pGIP212.20 and pGIP212.21. The *PstI* site was created by PCR amplification (see 5′ primer in panel A). The small vertical arrow indicates the position of the signal peptidase cleavage site.

MRS medium plates containing erythromycin. Of 12 clones, 5 had a stable resistance phenotype greater than 90%, compared with less than 50% for the other 7 clones. At this stage, we assumed that in the stable clones the entire plasmid had integrated into the chromosome (strains Lp801 and Lp802 [Fig. 6]).

The next step was to select intrachromosomal recombination events between the direct repeats generated after plasmid integration. Recombination between the two smaller repeats is shown in Fig. 6. Recombination between the two smaller repeats could be distinguished easily from recombination between the two larger repeats since the resulting clones were Amy^+ Em^s, compared with the Amy^- Em^s phenotype observed after recombination between the two larger repeats. Thus, the Lp801 and Lp802 strains were cultured in liquid MRS medium for 30 generations with no selection. Dilutions of these cultures were spread onto MRS medium plates, and the colonies were velvet replicated onto starch-THB medium and erythromycin-starch-THB medium plates to distinguish between Amy^+ and Amy^- colonies. Between 35 and 55% of the colonies were Em^s, and only 10 to 25% of these colonies were Amy^+; the Amy^+ phenotype of these strains (designated Lp811 and Lp812, respectively) (Fig. 6) was 100% stable after culture for 100 generations in MRS medium.

Integration of the amylase construction into the *cbh* genes of Lp811 and Lp812 was confirmed by Southern blotting. The *Hin*dIII fragment containing the *cbh* gene (8) was larger in both disrupted strains than in the wild-type strain (6.4 kb, compared with 4.4 kb in wild-type strain Lp80), confirming that the 2.0-kb amylase construction was inserted without any additional rearrangement (Fig. 6B). PCR amplification was performed to confirm the orientation of the amylase construction in Lp811 and Lp812. The positions of the primers are indicated in Fig. 6. PCR fragments of the diagnostic sizes (1.2 kb for primers 1 and 2 in Lp811 and 0.5 kb for primers 2 and 3 in Lp812) were obtained as expected (data not shown).

The levels of amylase in the culture medium after 16 h of growth in THB medium were 8.0 U/liter for Lp811 and 4.9 U/liter for Lp812. This twofold difference most likely resulted from the opposite orientations of the amylase construction, since transcription of the amylase insert could also be driven by the *cbh* promoter in Lp811. For comparison, amylase production was measured in the same strain transformed with the same gene construction but carried by an autoreplicative plasmid. Surprisingly, strain Lp80(pGIP212.1) produced 4.7 U of amylase per liter, suggesting that the autoreplicative plasmid had a low copy number in this strain or that a favorable position effect, even in the opposite direction of transcription, was conferred on the insert at the *cbh* locus.

DISCUSSION

The use of *L. plantarum* for heterologous production of exoenzymes has been proposed previously in the context of silage by workers in different laboratories (2, 17, 39, 40). Genes encoding α-amylase (*Bacillus stearothermophilus*, *Bacillus amyloliquefaciens*), xylanase (*Clostridium acetobutylicum*, *Clostridium thermocellum*), β-glucosidase (*C. thermocellum*), and cellulase (*C. thermocellum*, *C. acetobutylicum*, *Butyrivibrio fibrisolicus*) have been cloned in *L. plantarum*. Production was very low in all cases, and sometimes only one orientation of the gene gave rise to expression or no activity at all could be detected. Of the 11 cellulase, xylanase, and β-glucosidase genes cloned into *L. plantarum* by Scheirlinck et al. (39), only 2 were capable of ensuring exoenzyme production at a detectable level in liquid medium. The reasons for poor expression

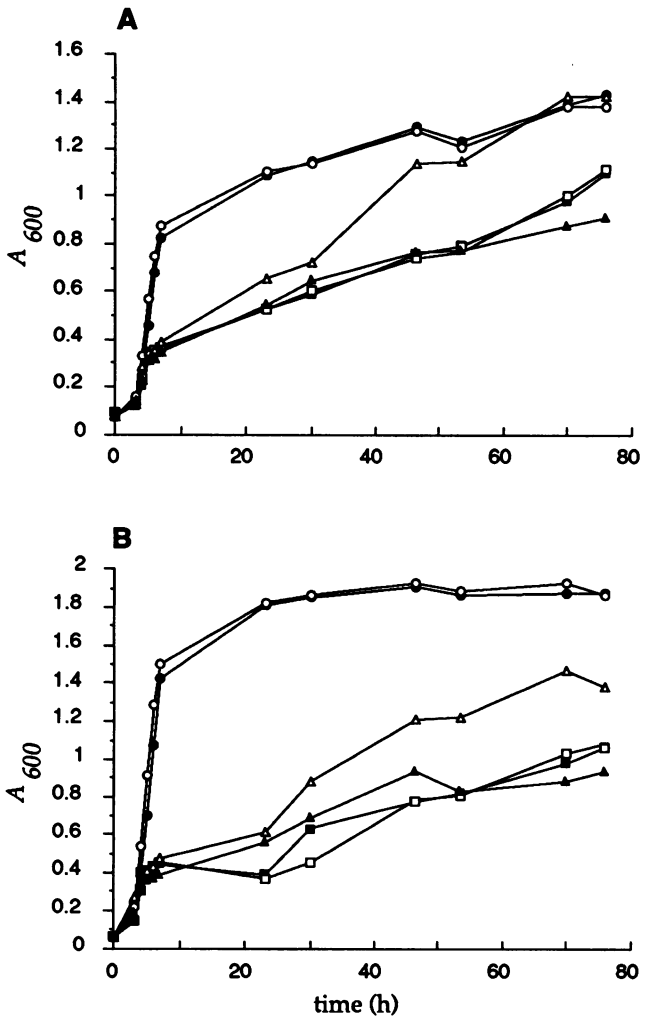

FIG. 5. Growth curves for wild-type *L. plantarum* strains and *L. plantarum* strains producing levanase in MRS media. (A) Strain NCIB8826. (B) Strain Lp80. Symbols: squares, MRS medium without glucose; circles, MRS medium supplemented with 2% glucose; triangles, MRS medium supplemented with 1% inulin; solid symbols, wild-type strain; open symbols, pGIP212.17-transformed strains.

were not investigated. Our data show that the *amyL* gene is no exception since it is also expressed at very low levels in *L. plantarum* because of a failure to recognize its proper promoter, as demonstrated by terminator insertion in front of the gene. The lack of *amyL* promoter recognition has been observed previously in *Enterococcus faecalis* (13), *Streptococcus salivarius* subsp. *thermophilus*, and *Lactococcus lactis* (44), confirming that the *amyL* gene carries a specific and/or regulated promoter which is not operative in various gram-positive bacteria outside the genus *Bacillus*. Replacement of the genuine *amyL* promoter by a strong *L. plantarum* promoter resulted in extracellular amylase production at a high level. This high level of expression and secretion (more than 90%) demonstrated that the RBS and signal peptide from this *Bacillus licheniformis* gene were compatible with the translation and secretion machinery of *L. plantarum*. A high level of secretion efficiency has also been reported with the *celE* (2) and *celD* (39) cellulase genes of *C. thermocellum* in the same species. Efficient secretion of α-amylase validates the use of the *amyL* gene as a reporter gene for the cloning of expression-secretion cassettes from the *L. plantarum* chromosome. The pGIP212 probe vector carrying the *amyL* gene deleted of its own expression and secretion signals was initially used to probe *Enterococcus faecalis* expression-secretion cassettes (13), and it has now been used successfully in *L. plantarum*.

Five different expression (transcription and translation) or expression-secretion cassettes were isolated from *L. plantarum* and sequenced. The cassettes containing the classical tripartite structure of a signal peptide provided a clearly distinguishable phenotype on plates, and amylase was secreted at efficiency levels between 60 and 90%. In contrast, cassettes from clones displaying low levels of starch degradation on plates did not contain any signal peptide, and amylase was located mainly intracellularly. The N-terminal peptides encoded by the true expression-secretion cassettes had all of the characteristics of signal peptides found in gram-positive bacteria. The extreme N terminal region (n region) and the hydrophobic core (h region) are longer than the regions found in signal sequences typical of gram-negative bacteria (43). More intriguing is the signal peptide from the pGIP212.12 cassette, which has all of the features of a lipoprotein signal peptide. If we assume that the cleavage site (SIA/C) is truly recognized by signal peptidase II, the hydrophobic core would be shortened to 11 amino acids, which makes the overall signal peptide structure similar to the structure of a true gram-positive lipoprotein exportation sequence (43). The extracellular production of amylase is less efficient in this case (60%) than in the two other expression-secretion cassettes. It is possible that part of the amylase precursor encoded by plasmid pGIP212.12 is processed as a lipoprotein and thus remains anchored to the cytoplasmic membrane or that the amylase preprotein exclusively follows the lipoprotein pathway and is subsequently partially released by extracellular proteases. This hypothesis could be tested by performing kinetic studies and also by replacing the cysteine present at the cleavage site.

None of the cassettes described in this paper exhibit any similarity with previously described exoenzymes. This observation raises the question of the origin of these inserts. A variety of signal peptides have been obtained from *Bacillus subtilis* (45), *Lactococcus lactis* (29, 42), and *Enterococcus faecalis* (13) by using different secretion probe vectors, and these peptides are not related to other previously described secreted proteins. It is possible that some of the randomly cloned sequences belong to membrane proteins containing signal peptides which could be cleaved or not (51, 53). In the case of uncleaved signal peptides, the N-terminal part of the integral protein is similar to a true signal peptide except that it does not contain a cleavage site. However, fusing this type of signal peptide to an unrelated reporter protein could lead to activation of a cryptic cleavage site, which could then be recognized by the signal peptidase, thereby allowing secretion (21).

Screening candidates on plates containing various media revealed an interesting glucose regulation phenomenon. Glucose repressed extracellular amylase production irrespective of the different expression or secretion signals. A fractionation experiment was conducted with NCIB8826(pGIP212.1) to evaluate the extent of this repression and to detect any potential intracellular amylase accumulation. It was surprising to observe that after 16 h of culture in liquid THB medium supplemented with 2% glucose, no activity was detected either extracellularly or intracellularly (data not shown). A specific regulation mechanism that acts selectively on putative regula-

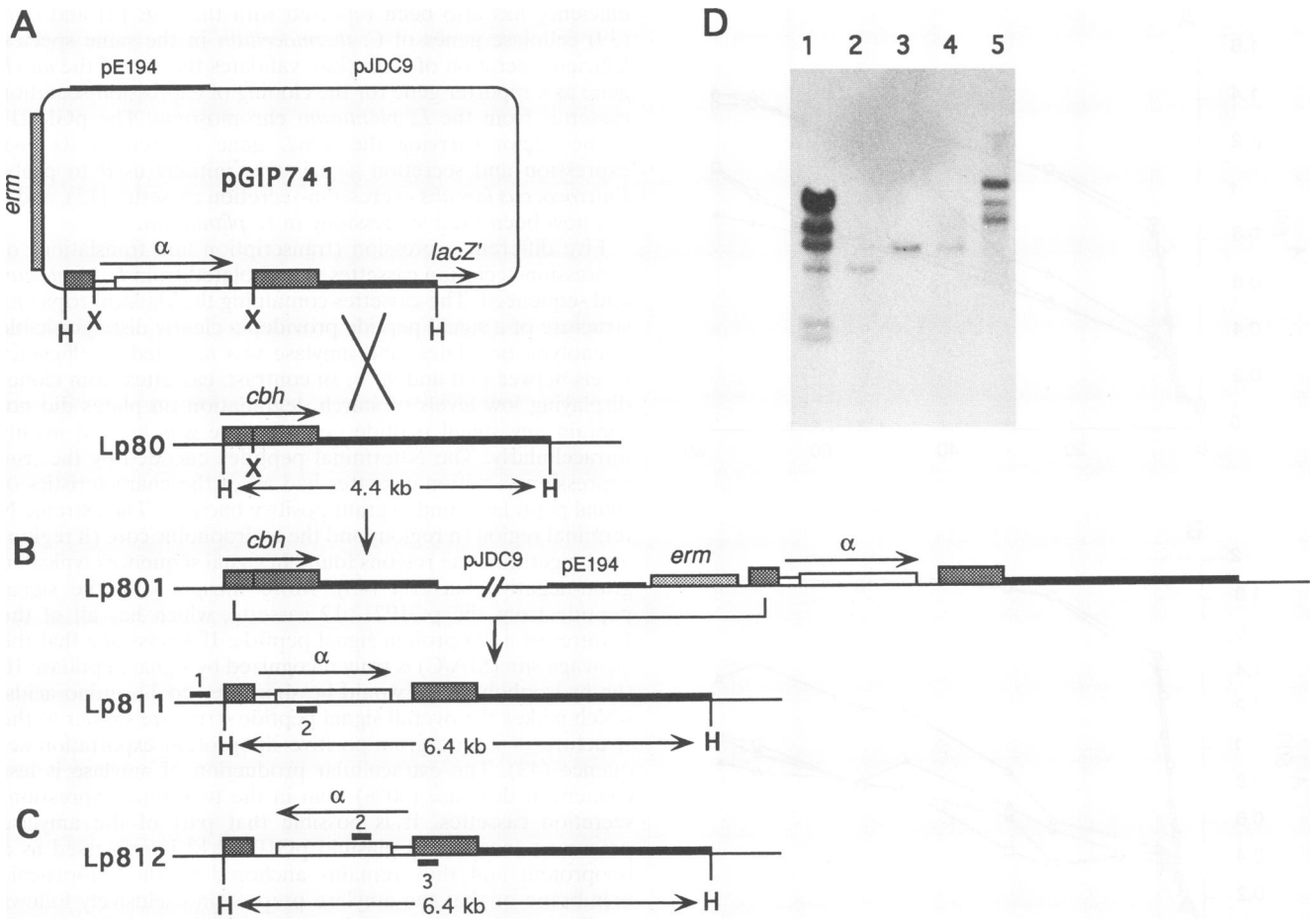

FIG. 6. Two-step stable chromosomal integration of the pGIP212.1 α-amylase construction in *L. plantarum*. (A) Homologous recombination between unstable autoreplicative plasmid pGIP741 and the Lp80 chromosome taking place in the larger 3′ moiety of the *cbh* gene to yield the Lp801 intermediate configuration at the *cbh* locus. (B) Intrachromosomal recombination between the tandemly repeated 5′ *cbh* fragments present at the site of integration in strain Lp801 to yield strain Lp811. This transgenic strain contains in the *Xba*I restriction site of the *cbh* gene a unique copy of the α-amylase construction in the same transcriptional orientation. (C) Construction of strain Lp812 by the same general strategy, using plasmid pGIP742 containing the amylase insert in the opposite orientation. The small black boxes (numbered 1, 2, and 3) indicate the positions of the PCR primers used to confirm the orientations of the amylase inserts in Lp811 and Lp812. The estimated sizes of the *Hin*dIII fragments for Lp80, Lp811, and Lp812 are indicated below the graphs. Symbols: H, *Hin*dIII; X, *Xba*I; α, amylase insert; *erm*, erythromycin resistance marker. (D) Southern blot hybridization of the ^{35}S-labelled *Hin*dIII fragment from pGIP741 (see panel A) to *Hin*dIII digests of chromosomal DNAs from Lp80 (lane 2), Lp811 (lane 3), and Lp812 (lane 4). Lane 1 contained phage λ digested with *Hin*dIII, and lane 5 contained undigested pGIP741.

tory signals from *L. plantarum*, *Enterococcus faecalis*, and *Bacillus licheniformis* is difficult to imagine. Each of our construction has the same ORF coding for the mature α-amylase, but when this ORF is replaced by the equivalent part of the *sacC* ORF through transcriptional or translational fusion, the same glucose effect is observed (data not shown). Conversely, the *amyL* and *sacC* genes both code for exoenzymes, and regulation more likely is at the cotranslational secretion level, such that in the presence of large amounts of a directly assimilable carbon source, secretion could specifically be blocked or reduced (38).

The translation initiation regions (TIRs) of the five different cassettes and of different genes isolated from *L. plantarum* were analyzed. All of these regions contain a strong SD sequence at an acceptable distance from the start codon (ATG, GTG, or TTG). These features are generally shared by all gram-positive bacteria, but there are reasons to believe that the presence of a SD sequence at a correct distance from a start codon is not sufficient to define a true translation start site (12, 16, 41). A number of additional interactions with different regions of the 16S rRNA have been suggested for *E. coli* (28, 30, 46, 49), and these interactions are always in the vicinity of the SD sequence (either upstream or downstream). An analysis of eight different TIRs from *L. plantarum* revealed the presence of conserved regions (5′ boxes) just upstream and downstream of the SD sequence which could interact with the 5′ end of the *L. plantarum* 16S rRNA. These putative interactions exhibited negative ΔG values which ranged from 4.8 to 13.8 kcal/mol (average, 8.0 kcal/mol). The possible role of the 16S rRNA 5′ end from *E. coli* was one of the first additional interactions described. It has been suggested (49) that this region could interact with complementary regions overlapping

the SD sequences of different TIRs from RNA coliphages which are known to be strongly recognized by *E. coli* ribosomes. More recently, a possible interaction with the 16S rRNA 5′ end has been identified downstream of the start codons of several *E. coli* TIRs (30). This additional interaction might enhance the affinity of mRNA for the 30S ribosomal subunit. Furthermore, reconstituted *E. coli* ribosomal 30S subunits lacking five nucleotides at the 5′ end of the 16S rRNA exhibited a 30% reduction in the ability to bind mRNA in vitro (27). It is conceivable that *L. plantarum* TIRs have evolved additional contacts between the ribosome and the mRNA. As the extreme 5′ end is divergent in different 16S rRNA sequences, we suspect that the putative 5′ boxes are recognized only by *L. plantarum* or a closely related species. In this context, it is interesting to recall that the majority of the *L. plantarum* cassettes were not able to produce high intracellular or extracellular levels of amylase in *Enterococcus faecalis*. It is difficult to determine whether the problem is at the transcriptional level or the translational level since the cassettes bear both types of genetic signals; however, it is possible that the lack of interaction with the 16S rRNA 5′ end from *Enterococcus faecalis*, whose sequence is known to be different from that of *L. plantarum* (19, 22), could prevent recognition of the *L. plantarum* TIRs by the 30S ribosomal subunits from *Enterococcus faecalis*. Conversely, the *Enterococcus faecalis* TIRs described so far (13) do not have such a high density of sequences complementary to the 5′ end of the *Enterococcus faecalis* 16S rRNA in the vicinity of the SD sequence. These motifs are shorter (not more than 5 consecutive nucleotides long) and are not found downstream of the SD sequence (data not shown). Additional experiments will involve the manipulation of these 5′ boxes in different ways to definitely assign an enhancing role to this putative interaction.

The ability of the cloned expression and secretion signals to efficiently drive expression and secretion of an exoenzyme other than amylase was tested by fusing different transcriptional and translational signals with the *Bacillus subtilis sacC* gene encoding levanase. All of the constructions obtained produced levanase at different rates, and the best producer in two different strains supported growth in a medium in which inulin was the major carbon source. Construction of recombinant *L. plantarum* strains that produce levanase is particularly interesting in the silage context since levans are the most abundant soluble carbohydrate polymers in temperate grasses (25).

In this study we identified a range of different homologous and heterologous transcription, translation, and secretion signals which are now available for improving expression of foreign genes in *L. plantarum*. The construction of recombinant strains for practical use requires stabilization of the gene(s) of interest. The main goal for food and feed applications is to achieve chromosomal integration of a foreign gene(s) devoid of any resistance marker or vector sequences in a manner that can be applied to industrial strains, which often can be transformed only at low levels of efficiency. Stabilization of cellulase and amylase genes in the *L. plantarum* chromosome has been obtained previously through Campbell-like integration with nonreplicative plasmids (40). This strategy requires a high transformation efficiency, and the recombinant locus is unstable because of the presence of direct repeats flanking the integrated gene of interest and moreover contains plasmid sequences that include a resistance marker gene. Another available strategy is the double crossover integration scheme that has been used for stable disruption of the *L. plantarum cbh* gene by a resistance marker (20). The two disadvantages of this alternative method are the requirement for a very high level of transformation efficiency and the requirement for coupling of a selection marker with the gene of interest. The method described in this paper solves most of these problems. Our method is a variant of a method used in previous work for the stabilization of an amylase construction in the chromosome of *Enterococcus faecalis* (14). The low transformation efficiency of industrial strains is bypassed through the use of an unstable replicative plasmid as described by Rixon et al. (35) for single crossover integration in *L. plantarum*. Stable integration of the foreign gene is obtained by a two-step procedure, in which each step can easily be selected through successive stabilization of the resistance marker followed by loss of this marker. This method allowed the complete stabilization of the pGIP212.1 amylase construction through disruption of the *L. plantarum cbh* gene. The *cbh* locus appeared to be particularly suitable with respect to the level of amylase production achieved by the integrated gene compared with the autoreplicative version. Not only was a comparable level of production of α-amylase maintained, a twofold increase was even observed when the amylase construction was in the same orientation as the *cbh* gene. This observation suggests that this amylase construction does not benefit from a high gene dosage effect with the vector used and/or that the *cbh* locus is rather favorable to transcription. On the other hand, the level of amylase or levanase production is lower in strain Lp80 than in NCIB8826. Similar strain-related differences have been reported previously by Sibakov et al. (42) in *Lactococcus lactis* strains, and these differences were correlated with the lysozyme resistance of the cell wall.

In summary, high-level expression of the *Bacillus licheniformis amyL* gene and the *Bacillus subtilis sacC* gene could be achieved easily in *L. plantarum* by using homologous transcription, translation, and secretion signals. We also found that some transcription, translation, and secretion signals from *Bacillus licheniformis*, *Bacillus subtilis*, and *Enterococcus faecalis* could be used efficiently for expression and secretion in *L. plantarum*. In contrast, we observed that most of the *L. plantarum* cassettes isolated are not recognized in *Enterococcus faecalis*. Furthermore, amylase and levanase production in *L. plantarum* is repressed by glucose through an as-yet-unexplained mechanism. Finally, an integration method was developed to stabilize heterologous genes in *L. plantarum* and this technique solves most of the problems encountered with the integration strategies developed previously for this species. Our work provides genetic tools which should be useful for the development of recombinant strains amenable to practical applications in the food and feed sector.

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