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Integrative Food-Grade Expression System Based on the Lactose Regulon of *Lactobacillus casei*

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The lactose operon from *Lactobacillus casei* is regulated by very tight glucose repression and substrate induction mechanisms, which made it a tempting candidate system for the expression of foreign genes or metabolic engineering. An integrative vector was constructed, allowing stable gene insertion in the chromosomal lactose operon of *L. casei*. This vector was based on the nonreplicative plasmid pRV300 and contained two DNA fragments corresponding to the 3′ end of *lacG* and the complete *lacF* gene. Four unique restriction sites were created, as well as a ribosome binding site that would allow the cloning and expression of new genes between these two fragments. Then, integration of the cloned genes into the lactose operon of *L. casei* could be achieved via homologous recombination in a process that involved two selection steps, which yielded highly stable food-grade mutants. This procedure has been successfully used for the expression of the *E. coli gusA* gene and the *L. lactis ilvBN* genes in *L. casei*. Following the same expression pattern as that for the lactose genes, β-glucuronidase activity and diacetyl production were repressed by glucose and induced by lactose. This integrative vector represents a useful tool for strain improvement in *L. casei* that could be applied to engineering fermentation processes or used for expression of genes for clinical and veterinary uses.

Lactic acid bacteria (LAB) have been used for centuries in the preparation and processing of foods and beverages. Due to its great economic importance for the agrofeed sector and its alleged importance for human and animal health, research on the characterization, metabolism, and genetics of the genus *Lactobacillus* has increased over the last decade. Several vectors have been developed to express genes and to secrete proteins in *Lactobacillus* (12, 26, 36, 37, 47, 48). However, if these vectors are to be considered safe for humans, animals, or the environment, only DNA from organisms generally regarded as safe should be used, and no antibiotic resistance markers should remain after genetic manipulation. The integration of foreign genes into the genome constitutes an interesting option for stably maintaining cloned genes without the need for selective markers. Technically, foreign gene integration could be achieved by homologous recombination through cloned DNA fragments (randomly cloned fragments or target genes) and by self-integrative elements (insertion sequences or an attachment site and integrase gene). In the genus *Lactobacillus*, stabilization of cloned genes is normally achieved by chromosomal integration, based on the use of cloned DNA fragments in nonreplicating plasmids. Stable chromosomal integration of the genes encoding the α-amylase from *Bacillus stearothermophilus* and a cellulase from *Clostridium thermocellum* was obtained in *Lactobacillus plantarum* using a randomly cloned chromosomal fragment as the integration target (42). A similar strategy was used to construct an integrative vector for *Lactobacillus acidophilus* (25). Other ingenious systems have been developed using a phage integrase-mediated site-specific insertion in the host chromosome (4, 6, 28). There are also examples of stable integration in target genes, such as *cbh*, which encodes a bile salt hydrolase, and *pep*XP, which encodes an X-prolyl-dipeptidyl aminopeptidase, from *L. plantarum* and *L. helveticus*, respectively (9, 22). All foreign genes integrated by these procedures are normally expressed from their own promoters, which makes more difficult the control of their regulation. The α-amylase gene from *Bacillus licheniformis* (*amyL*) was satisfactorily expressed in *L. plantarum* only when the *amyL* promoter was replaced by an *L. plantarum* promoter (42).

Very efficient expression systems based on antimicrobial peptide (nisin), sugar utilization, or nonsense suppressors have been developed for *Lactococcus lactis* (12, 14, 23, 40). However, besides the nisin system, these approaches could not be transferred to species of *Lactobacillus*. In lactobacilli, the regulation of gene expression has been studied mainly for carbon catabolism pathways, such as those of lactose, xylose, ribose, sorbose, and arginine deiminase (1, 2, 3, 10, 18, 19, 30, 34, 35, 43, 49, 50). In *Lactobacillus casei*, the best-characterized sugar transport is the lactose-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS). The *lac* operon, *lacTEGF*, encodes an antiterminator protein (LacT), lactose-specific PTS proteins (LacE and LacF), and a phospho-β-galactosidase (P-β-Gal) (LacG) (1, 2, 3, 18, 34). It has been previously reported (3, 19, 30) that the expression of the *lac* operon in *L. casei* ATCC393 (pLZ15$^-$) is subject to dual regulation: carbon catabolite repression (CCR) mediated by the general regulator CcpA and induction by lactose through transcriptional antitermination. LacT, whose activity is modulated by the EII elements of the lactose PTS (LacE and/or LacF), mediates the latter mechanism. Furthermore, HPr, a general component of PTS, and LacT are involved in an additional CcpA-independent CCR effect (19, 39, 44).

In this report, an integrative expression vector that allowed the selection of stable mutants that express *Escherichia coli gusA* and *L. lactis ilvBN* genes through the lactose regulon is described. This integrative vector represents the first specific

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
L. casei CECT[a] 5275	Wild type	B. Chassy (University of Illinois, Urbana)
L. casei CECT 5276	*L. casei* CECT 5275 with a frameshift in *lacF*	19
L. casei CECT 5290	*L. casei* CECT 5275 with *gusA* gene integrated	This work
L. casei CECT 5291	*L. casei* CECT 5275 with *ilvBN* genes integrated	This work
L. lactis MG1363	Source of *ilvBN* genes	16
Plasmids		
pRV300	Er^r from pAMβ1	24
pNZ272	*gusA* Cm^r	33
pIlacF	*lacF* gene cloned as *Eco*RV/*Kpn*I fragment in pRV300	This work
pIlac	*lacG* 3′ end and *lacF* gene in pRV300	This work
pIlacgus	pIlac containing *gusA* gene	This work
pIlacilv	pIlac containing *ilvBN* genes	This work

[a] CETC, Colección Española de Cultivos Tipo.

expression system developed for *L. casei* that has great potential for food industry and health applications.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this work are listed in Table 1. *L. casei* cells were grown in MRS medium (Oxoid) and MRS fermentation broth (Adsa-Micro; Scharlau S.A., Barcelona, Spain) plus 0.5% of the different carbohydrates at 37°C under static conditions. *E. coli* DH5α was grown with shaking at 37°C in Luria-Bertani medium. Plating of bacteria was performed on the respective media solidified with 1.5% agar. When required, the concentrations of antibiotics used were 100 μg of ampicillin per ml to select *E. coli* transformants and 5 μg of erythromycin per ml for *L. casei*.

Recombinant DNA procedures. Genomic DNA from *L. casei* and *L. lactis* strains was purified using a Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, Minn.), following the procedure described by the manufacturer. Restriction and modifying enzymes were used according to the recommendations of the manufacturers. General cloning procedures were performed as described by Sambrook et al. (41). *L. casei* was transformed by electroporation with a gene-pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.). For Southern blot hybridization, *L. casei* DNA was digested with *Bgl*II and *Pst*I endonucleases, separated on agarose gel, and blotted to a Hybond nylon membrane (Amersham). The probes used in the three hybridization experiments were Perm, Pilv, and Plac. The Perm probe consists of an *erm* gene from the pRV300 plasmid digested with *Bam*HI endonuclease. Pilv corresponds to *ilvBN* genes from *L. lactis* that were obtained by PCR using ilv1 and ilv2 oligonucleotides as primers (for sequence, see below), with the genomic DNA of a *Lactococcus* strain as a template. The Plac probe comprised a fragment of 537 bp that corresponds to the 3′ end of the *lacG* gene. This DNA fragment was obtained by PCR using genomic DNA from *L. casei* as a template and the oligonucleotides lac46 (5′TGCGTGCCTATCATGGC) and lac6 (5′CTTGCTGTCTAAATAGCC) as primers. The DNA probes were prepared using the reagents from the Boehringer digoxigenin-DNA labeling kit as recommended by the manufacturer. Hybridization, washing, and staining were done as described by the supplier. PCR was performed using the Expand High fidelity PCR system (Roche Molecular Biochemicals), containing 200 μM concentrations of each deoxynucleoside triphosphate and 10 pmol of each primer. Upon agarose gel electrophoresis, the amplified DNA was recovered with the GFX PCR kit (Amersham Pharmacia Biotech).

Construction of integration plasmids. The integrative vector, pIlac, is based on the vector pRV300 (24), which does not replicate in *Lactobacillus*, and it carries the *erm* gene from pAMβ1. This vector was constructed in a two-step cloning experiment. A 461-bp DNA fragment containing the *lacF* gene was amplified by PCR from an *L. casei* chromosome with the primers lac43 (5′TACATATGCCCGGGGAATTCAATCGGAGGGAAAATG) and lac45 (5′TTGAGGTACCGCTAACAGC). The Lac43 primer showed several substitutions (boldface) to introduce the new *Nde*I, *Sma*I, and *Eco*RI sites (underlined) in the 45-bp region between *lacG* and *lacF*. Lac45 contained three substitutions (boldface), generating a new *Kpn*I site (underlined). The blunt-ended fragment amplified was digested with *Kpn*I and cloned into pRV300 that had been previously digested with *Eco*RV and *Kpn*I. This construction, pIlacF, was used to clone an 878-bp DNA fragment, containing the 3′ end of the *lacG* gene and 23 bp of the 45-bp intergenic region between the *lacG* and *lacF* genes. This fragment was amplified by PCR from an *L. casei* chromosome using the primers lac49 (5′ATAAGAGCTCCCAAGCTGA) and lac42 (5′TGCATATGCTGCAGCCTCCTTTTAATCCGGAATG). Lac49 had three substitutions (boldface), generating a *Sac*I site (underlined). The Lac42 primer contained several substitutions (boldface), creating new *Nde*I and *Pst*I sites and a ribosome-binding site (RBS) (underlined). Then this fragment was cloned into the *Nde*I/*Sac*I sites of pIlacF. Fig. 1A shows the physical map of the resulting integrative vector, pIlac.

A DNA fragment containing the *E. coli gusA* gene was amplified by PCR using the plasmid pNZ272 (33) as a template and the primers gus1 (5′AAAACTGCAGTATTATTATCTTAATGAGG) (a newly created *Pst*I site is underlined) and gus2 (5′CGGAATTCTCATTGTTTGCCTCCC) (a newly created *Eco*RI site is underlined). The amplified DNA fragment was purified, digested with the endonucleases *Eco*RI and *Pst*I, and ligated into *Eco*RI/*Pst*I-digested pIlac. This construction was named pIlacgus (Fig. 1B).

The *ilvBN* genes were amplified from the genomic DNA of *L. lactis* using the oligonucleotides ilv1 (5′CGATCATATGAAAAAAATAAAGTTAGAAAAACCTACTTCC) and ilv2 (5′CCGAATTCTTAGCCACGCTCAAAACCTGC) as primers, containing *Nde*I and *Eco*RI sites (underlined), respectively. The amplified fragment was cloned into *Nde*I/*Eco*RI-digested pIlac to give pIlacilv (Fig. 1B).

Enzymatic assays. P-β-Gal and β-glucuronidase activities were assayed as previously described (33, 46) in permeabilized *L. casei* cells.

Total nitrogen determination. The procedure used was based on the method described by Doi et al. (15).

Determination of end metabolites. The metabolites released by wild-type and mutant strains grown on glucose plus lactose, lactose, or ribose have been analyzed in a resting cell system (11). The analysis of volatile compounds such as ethanol, acetaldehyde, acetone, 1-butanol, acetoin, and diacetyl was performed using a purge-and-trap apparatus equipped with a Vocarb 3000 trap (Supelco) to concentrate the analytes and coupled to a gas chromatographer equipped with a mass spectrometer (Hewlett-Packard 7695) (Barcelona, Spain) as described by Dauneau et al. (11). The α-acetolactate (ALA) determination is based on its oxidative decarboxylation to diacetyl as previously reported, with some modification (38). The samples (2 ml each) were pretreated in a 4-ml vial by addition of 150 μl of 1.85 M $FeCl_3$ and 1 ml of 80% lactate buffer (pH 2.8) and vigorously stirred for 5 s. The vials were hermetically sealed with teflon-lined rubber seals and heated at 75°C for 30 min. The concentration of ALA was calculated by subtraction of the diacetyl concentrations found before and after decarboxylation of the samples.

Lactic acid produced in the resting cell system was measured with a D-lactic acid/L-lactic acid enzymatic bioanalysis kit (Boehringer-Mannheim) as described by the supplier. The total amount of lactic acid produced corresponds to the addition of the concentrations of both isomers determined.

RESULTS

Integration strategy with vector pIlac. This vector (Fig. 1A) contains two regions of homology that are physically close in the *L. casei* chromosome, a 3′ fragment of *lacG* (Δ*lacG*) and *lacF*. The sequence of the intergenic region has been modified, keeping intact the spacing between both genes, to introduce a typical RBS for *Lactobacillus* and a multiple cloning site (*Pts*I, *Nde*I, *Sma*I, and *Eco*RI), allowing the cloning of new genes, so that after integration, the transcription of these genes would take place from the *lac* promoter and the newly created RBS would facilitate their translation initiation.

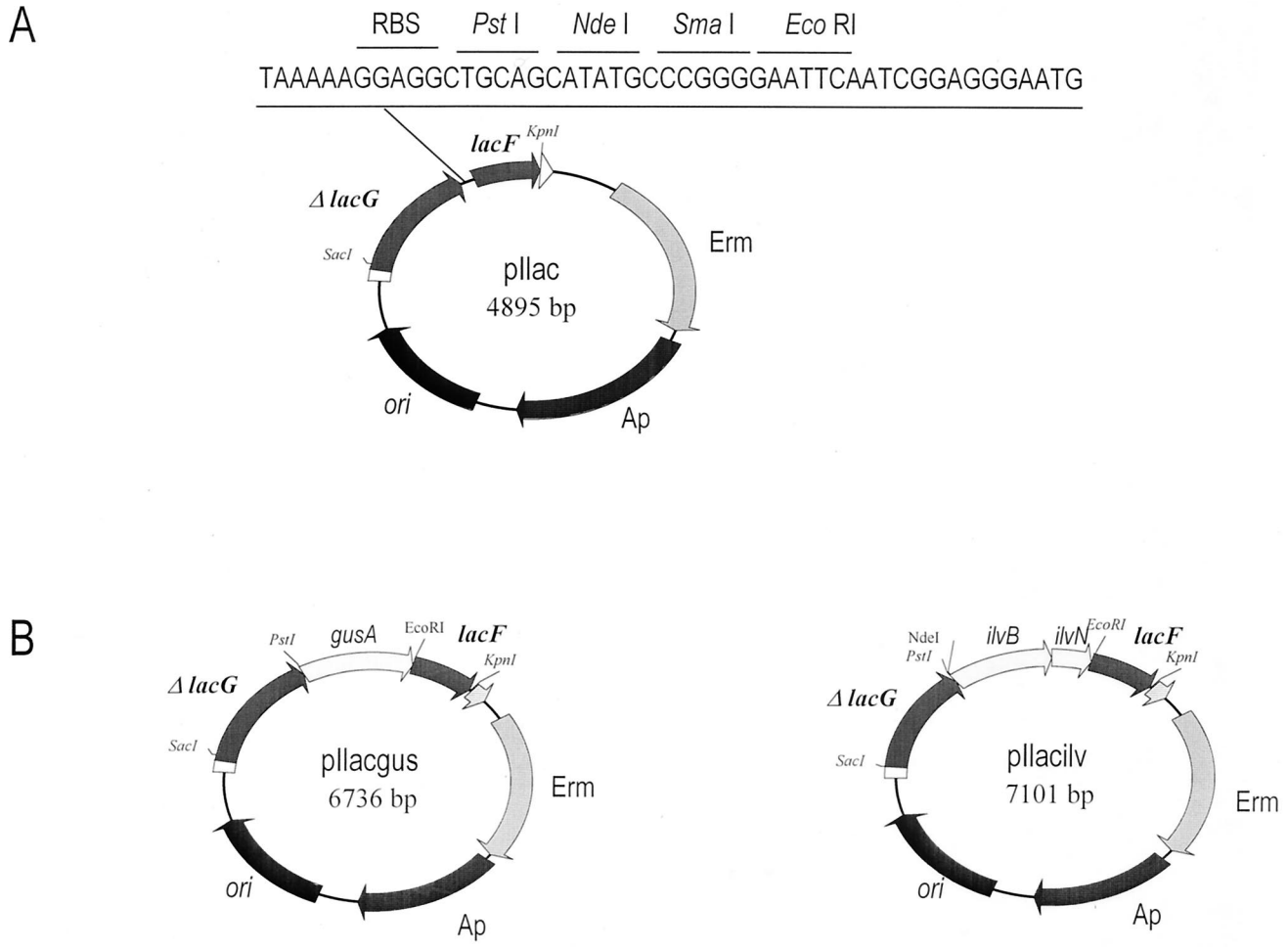

FIG. 1. Restriction maps of integrative vectors. (A) Integrative vector pIlac. Erm and Ap are erythromycin and ampicillin resistance genes: *ori* represents the *E. coli* replicon. *lacG* and *lacF* genes encode P-β-Gal and $EIIA^{lac}$ of the *lac* operon. The 45 bp of intergenic region are shown with the newly created restriction sites and an RBS. (B) Vector pIlacgus. The *gusA* gene was cloned into *Pst*I/*Eco*RI pIlac. Vector pIlacilv was constructed by cloning *ilvBN* genes into *Nde*I/*Eco*RI pIlac.

For the integration of the cloned genes in the chromosome, two recombination events should take place, one in each of the homologous regions (Δ*lacG* and *lacF*). The use of a *lacF* frameshift mutant (19), *L. casei* CECT 5276, as the host strain (Lac^-) facilitated the selection of the clones that had undertaken the second recombination as Lac^+ clones among the Lac^- background, with the following procedure. After electroporation of *L. casei* CECT 5276 with pIlac derivatives, Lac^+ Er^+ and Lac^- Er^+ transformants were recovered, depending on the region where the Campbell-like recombination had occurred. A transformant with the Lac^- Er^r phenotype was grown for 200 generations in MRS broth without antibiotic in order to allow the second recombination event, which would excise the plasmid rendering Lac^+ Er^s colonies (one out of 20 viables). This strategy can be applied for the insertion of any gene of interest in the *lac* operon of *L. casei* and rendered mutants totally deprived of any DNA sequence from the plasmid and *erm* gene.

Chromosomal integration of *E. coli gusA* into *L. casei*. In order to evaluate the potential of the integrative vector pIlac as a vehicle for chromosomal gene insertion, the β-glucuronidase-encoding gene of *E. coli*, *gusA*, was cloned into it. The plasmid obtained, pIlacgus (Fig. 1B), was used to transform *L. casei* CECT 5276. Following the procedure described above, colonies that had undergone a second recombination event suffered the excision of the vector, giving rise to Erm^s Lac^+ colonies which had the *gusA* gene integrated into the *lac* operon. The first and second recombination events were confirmed by Southern blot hybridization of the integrants' chromosomal DNA (data not shown). The resulting new structure of the *lac* operon contained *gusA* between *lacG* and *lacF*, and as a consequence, the expression of *gusA* was subject to the same regulation as the *lac* genes. This was confirmed by measuring β-glucuronidase activity in one of the colonies selected, *L. casei* CECT 5290, when it was grown on ribose, lactose, and glucose plus lactose (Table 2). Greater P-β-Gal activity was detected for the *gusA* integrant on lactose than for the wild type and *ilvBN* integrant (described below), possibly due to the partial cleavage of ONPG-6-P (the P-β-Gal substrate) by β-glucuronidase. It could also be noticed that the growth rate of both integrants on glucose was identical to that of the wild type (data not shown). On lactose, duplication times were not substantially different during early growth stages (95.5 ± 2.6 min, 91.8 ± 3.4 min, and 90.3 ± 5.4 min for wild-type, CECT 5290, and CECT5291 strains, respectively); however, it was observed that both mutants (*gus* and *ilvBN*) would reach only an optical density at 550 nm of 0.8.

TABLE 2. Enzymatic activities in different strains of *L. casei*[a]

Strain	Relevant genotype	Sugar	Activity (nmol min^{-1} mg^{-1} dry wt)	
			P-β-Gal	β-glucuronidase
CECT 5275	Wild type	Ribose	2.03 ± 0.86	0.98 ± 0.15
		Lactose	23.91 ± 2.93	1.89 ± 0.19
		Glucose + lactose	0.78 ± 0.24	1.16 ± 0.23
CECT 5290	*gusA*	Ribose	10.040 ± 1.69	43.13 ± 11.64
		Lactose	57.84 ± 8.51	119.05 ± 8.91
		Glucose + lactose	1.26 ± 0.12	1.23 ± 0.2
CECT 5291	*ilvBN*	Ribose	6.43 ± 0.63	
		Lactose	27.11 ± 3.56	
		Glucose + lactose	1.56 ± 0.26	

[a] The values and standard deviations are from at least three independent experiments.

Construction of a food-grade *ilvBN* integrant of *L. casei*. Diacetyl is an important compound related to the characteristic flavor of many fermented milk products. Only a few LAB could produce this metabolite from the citrate of milk. During citrate fermentation, ALA synthase converts pyruvate to ALA, which could be converted spontaneously to diacetyl in the presence of oxygen. The *L. lactis ilvBN* genes encode the catalytic and regulatory subunits of acetohydroxy acid synthase (17). This enzyme is involved in biosynthesis of branched-chain amino acids, isoleucine and valine, converting pyruvate to ALA with higher affinity for pyruvate than ALA synthase. In this work, in order to increase the cellular pool of ALA that could be turned into diacetyl by oxidative decarboxylation, *ilvBN* genes of *L. lactis* were integrated into the chromosome of *L. casei*. Both genes were cloned into pIlac to give pIlacilv (Fig. 1B), which was used to transform *L. casei* CECT 5276. The selection strategy for recombinant colonies was identical to that described above. A double recombinant (Er^s and Lac^+) mutant of *L. casei* (*ivlBN* integrant) was selected for further analysis and designated *L. casei* CECT 5291. The pattern of P-β-Gal activity in this strain was similar to that in the wild-type strain, since it also is induced by lactose and repressed by glucose (Table 2).

Integration of the *ilvBN* genes into the chromosomal *lac* operon of *L. casei* was confirmed by Southern hybridization using the probe Plac, corresponding to the 3′ end of *lacG* (Fig. 2A). A hybridization band was detected on the genomic DNA of the *L. casei* CECT 5276 (host strain), the Er^r Lac^- integrant of pIlacilv (first recombination event), and *L. casei* CECT 5291 (double recombinant) (Fig. 2A, lanes 1, 2, and 3, respectively) when digested with *Bgl*II/*Pst*I. The fragment detected in *L. casei* CECT 5276 was larger than in the double recombinant because the fragment integrated containing *ilvBN* carries an additional *Pst*I site. The chromosomal integration of *ilvBN* was confirmed using a Pilv probe (Fig. 2B). Evidence that the antibiotic resistance gene (*erm*) had been excised from the *L. casei* genome was demonstrated using a Perm probe. In this Southern blot, the hybridization signal was detected only with the genomic DNA from the first integrant (Fig. 2C). Moreover, the *ilvBN* genes remained stably integrated on the genome after 50 overnight transfers in MRS medium without selective pressure (data not shown).

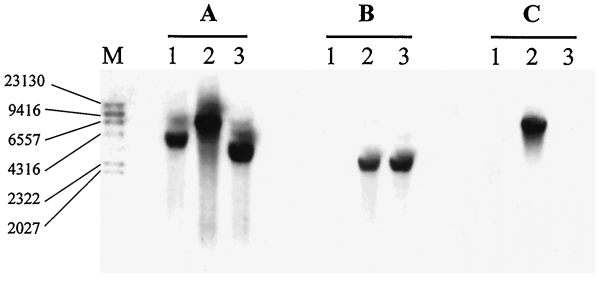

FIG. 2. Southern blot of total DNA digested with *Bgl*II/*Pst*I from *L. casei* CECT 5276 (lane 1), an integrant of pIlacilv (lane 2), and *L. casei* CECT 5291 (food-grade integrant) (lane 3). The probes used were Plac (A), Pilv (B), and Perm (C). M represents digoxigenin-labeled λ phage DNA digested with *Hin*dIII as a molecular size marker.

Determination of metabolic products in the *ilvBN* mutant. Metabolites released by the *L. casei* wild type and *L. casei* CETC 5291, carrying the integrated *ilvBN* genes in the lactose operon (*ilvBN* integrant), have been analyzed in a resting cell system when cells were grown on glucose plus lactose, lactose, and ribose (Fig. 3). Besides lactic acid, which is by far the most abundant compound, ethanol and acetone were the predominant metabolites accumulated by cells grown on ribose, on which *L. casei* becomes heterofermentative. Remarkable differences could be noticed in the *ilvBN* integrant on lactose, regarding the production of ethanol, 1-butanol, acetoin, and diacetyl. In particular, the amount of diacetyl accumulated by the lactose-induced *ilvBN* integrant was 23-fold greater than that for the wild type. Other significant differences found in the *ilvBN* integrant are related to the lower level of accumulation of ALA and lactate on lactose, possibly due to the diversion of pyruvate and ALA towards the synthesis of branched-chain amino acids (isoleucine, leucine, and valine). In order to test this hypothesis, total soluble nitrogen (amino acids) was determined in the supernatant of the resting cell systems on lactose, obtaining 1.25 ± 0.07 mM concentrations for the wild type, and 2.01 ± 0.26 mM concentrations for the *ilvBN* integrant. This difference (0.76 mM) could partially be explained by the secretion of a proportion of the excess amino acids synthesized by the integrant. Regarding acetaldehyde production, only small differences were observed between the two strains with different carbon sources. Unexpectedly, slightly higher concentrations of acetaldehyde and 1-butanol could be observed when the *ilvBN* integrant was grown on glucose plus lactose. This different behavior was clearly related to the presence of the *ilvBN* genes, indicating that some degree of expression of the acetohydroxy acid synthase was taking place on glucose plus lactose, altering the proportions in metabolites derived from acetyl coenzyme A.

DISCUSSION

The present study describes the construction of an integrative expression vector for *L. casei* that allowed the obtainment of stable food-grade integrants capable of expressing foreign genes under the tight control of the well-characterized *lac* operon promoter (Fig. 1A). Lactose genes have been used in other LAB for different biotechnological purposes, such as the construction of food-grade vectors in *L. lactis*, addressing integration in *Lactobacillus helveticus*, and gene expression in *Streptococcus thermophilus* (9, 27, 29, 31). However, both structural organization and regulation of the *lac* operon in *L. casei* are very different from those described for the other LAB (1, 2, 3, 13, 18, 19, 30, 34); it displays very tight glucose repression and lactose induction mechanisms, which were very promising for the expression of foreign genes. The integrative vector designed in this work, pIlac, allowed cloning of DNA fragments between the two target genes (Δ*lacG* and *lacF*). Then, through Campbell-like recombination, the genes of interest could be inserted in the *lac* operon, obtaining a food-grade

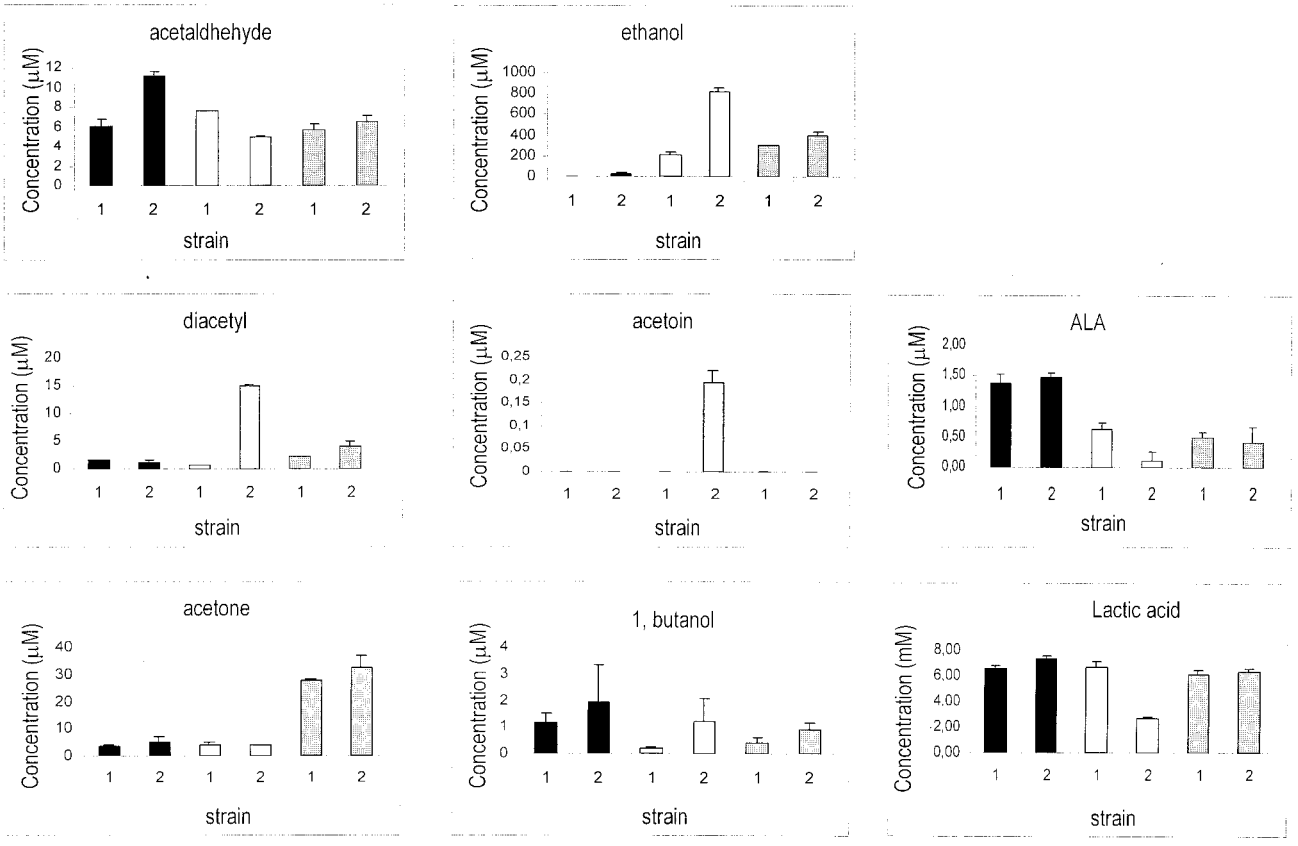

FIG. 3. End metabolite production (acetaldehyde, ethanol, diacetyl, acetoin, ALA, acetone, 1, butanol, and lactic acid) by the wild type (1) and *ilvBN* integrant (2) from cells grown on glucose plus lactose (black), lactose (white), and ribose (grey). The values are from at least three independent experiments, and the coefficient of variation for each mean was less than 10%.

construct in which the foreign genes became a functional part of the operon and were subject to the same regulation (Table 2). However, it could be observed that the insertion of foreign genes, *gusA* and *ilvBN*, led to some induction of the operon on ribose, as was shown by P-β-Gal and β-glucuronidase activities (Table 2). In a previous work it was shown that mutants in the genes encoding either of the lactose-specific PTS elements (*lacE* and *lacF*) showed constitutive transcription of the *lac* operon (19). It could be tentatively proposed that transcription of the new constructions gave a longer, more unstable mRNA, where perhaps *lacF*—placed at the end of it—could be less efficiently translated. This could be confirmed by the fact that neither of the integrants could grow on lactose for more than a few generations, possibly because of an inefficient lactose-PTS transport that allowed growth only at high lactose concentrations.

L. casei is frequently used as the starter culture in many fermentation processes, especially in cheese making or, recently, as a probiotic in fermented milk products. However, *L. casei* is not a good producer of diacetyl, and this is a very desirable compound in dairy fermentations. This product is normally synthesized in LAB from the glycolytic intermediate pyruvate, which is converted to ALA by the ALA synthase. Then, ALA is transformed to acetoin through ALA decarboxylase activity or to diacetyl in the presence of oxygen. Also, acetoin yields diacetyl by the action of acetoin reductase. Different approaches have been used with *L. lactis* to improve diacetyl production, such as deletion of acetolactate decarboxylase, mutation of lactate dehydrogenase, or overexpression of acetohydroxy acid synthase (8, 20, 21, 32, 45). This biotechnological approach was also considered in the present work. Induction of *ilvBN* genes by lactose in the food-grade system developed in this work yielded, in 3 h, a total amount of diacetyl comparable to that in overnight cultures of *L. lactis* overexpressing *ilvBN* (7, 8, 20, 45). However, further optimization of the diacetyl production through detailed fermentation studies could be achieved because, in our resting cell system, the high cell density and static incubation conditions in a sealed tube were possibly generating an adverse environment—poor in oxygen—where a lower diacetyl reductase activity favored a certain amount of accumulation of acetoin.

Another objective of this work was the evaluation of the balance of metabolites during the overexpression of "cross-road" enzymes, such as acetohydroxy acid synthase (encoded by *ilvBN*). The accumulation of ALA on glucose-grown cells suggests that the biosynthesis of diacetyl from this ketoacid could be subject to CCR. However, a major interference with an even greater overproduction of diacetyl in *L. casei* CETC 5291 can be attributed to the fact that pyruvate is also a substrate of acetohydroxy acid synthase in the anabolic pathway of branched-chain amino acids. Furthermore, both compounds, pyruvate and ALA, could be inducers of subsequent steps in these pathways (5), for which the overexpression of *ilvBN* was probably leading to a drainage of pyruvate for the synthesis of leucine, isoleucine, and valine. This was demonstrated by the smaller amount of lactate and higher concentration of amino acids detected in the supernatant of the lactose-induced mu-

tant during the resting cell assay. However, a metabolite balance could not be calculated. This is an intricate part of the metabolic map, and many more compounds should be analyzed to get a clearer picture of the carbon fluxes at this metabolic level.

The kind of experiment described in this work has never before been performed with lactobacilli. Due to its food-grade nature, the system developed here has a great potential for the metabolic engineering of intracellular metabolites and the production of different enzymes during dairy fermentation. However, due to the regular presence of lactobacilli in higher vertebrate mucosae, other applications could be envisaged, such as the delivery of antigens in the gut, mouth, or vagina as well as a variety of clinical and veterinary products.

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