Two genes encoding the β -galactosidase of *Lactobacillus sake*

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The β -galactosidase of *Lactobacillus sake* DSM 20017 is encoded by two genes located on its chromosome. These genes designated *lacL* and *lacM* were cloned in *Escherichia coli* NM 554 on an 8.65 kbp *HindIII* fragment inserted in vector pRB473. Deletion analysis of the originally cloned fragment revealed that both genes are required for the formation of a functional β -galactosidase. *lacL* and *lacM* are transcribed as a single transcript of approximately 2.9 kbp starting 34 bp upstream of the translational start codon. The proteins derived from *lacL* and *lacM* share only 18–59% homology with other β -galactosidases. The genes encoding the β -galactosidase are scattered with multiple direct and inverted repeats of 9–12 bp. However, comparison with the plasmid-encoded *Leuconostoc lactis* β -galactosidase revealed equal distribution of conserved amino acid residues and suggests that the genes have a common origin. Specific deletions or insertions resulting from the presence of the repeats were not observed. The *L. sake* β -galactosidase was phenotypically expressed in *E. coli* NM 554 and *Lactobacillus curvatus* LTH 1432. Its two genes can be used to replace antibiotic reporter genes to develop food-grade vectors and α -complementation systems for self-cloning in meat lactobacilli.

Keywords: *Lactobacillus sake*, lactose, β -galactosidase, cloning, sequence

INTRODUCTION

In sausage fermentations, strains of *Lactobacillus sake* and *Lactobacillus curvatus* are applied as starters to control the process. They gain dominance within the meat-borne microflora and ensure hygienical safety and a high-quality sausage (Hammes *et al.*, 1990). These strains should not effectively ferment lactose as this sugar is added as a water-binding agent at concentrations of up to 6 g kg⁻¹. In previous studies we have observed that the phospho- β -galactosidase (phospho- β -gal) activity in *L. sake* and *L. curvatus* is repressed by glucose, whereas β -galactosidase (β -gal) activity present in strains of *L. sake* was constitutive (Obst *et al.*, 1992). These activities were low as compared to those of lactic acid bacteria (LAB) in dairy fermentations.

In contrast to this limited knowledge on the lactose metabolism of LAB prevailing in meat fermentations, the

respective metabolic pathways are well studied in dairy LAB. The transport of lactose (Romano *et al.*, 1987; Hengstenberg *et al.*, 1989), splitting of the disaccharide (Farrow, 1980), the generation of phosphorylated sugar-derivatives and the regulation of the pathway (Chassy & Thompson, 1983) has been investigated thoroughly, especially with *Lactococcus lactis* subsp. *lactis*. Two systems for lactose transport and hydrolysis have been described which use a phosphotransferase system and a phospho- β -gal or a lactose permease in combination with a β -gal (McKay *et al.*, 1970). The genetic organization of enzymes involved in lactose metabolism and in some cases their regulation has been studied in lactococci (Boizet *et al.*, 1988; de Vos & Simons, 1988; de Vos *et al.*, 1990; van Rooijen, 1993), streptococci (Herman & McKay, 1986; de Vos & Simons, 1988), lactobacilli (Flickinger *et al.*, 1986; Shimizu-Kadota, 1988; Schmidt *et al.*, 1989; Leong-Morgenthaler *et al.*, 1991) and leuconostocs (David *et al.*, 1992). Furthermore, genes of the lactose metabolism have been used as markers to construct food-grade vectors (Hashiba *et al.*, 1992).

The β -gal genes of dairy LAB such as *Lactobacillus delbrueckii* subsp. *bulgaricus* (Schmidt *et al.*, 1989) or *Lactobacillus casei* (Flickinger *et al.*, 1986) and *Leuconostoc lactis* (David *et al.*, 1992) were found to be encoded

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Abbreviations: β -gal, β -galactosidase; LAB, lactic acid bacteria; phospho- β -gal, phospho- β -galactosidase.

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chromosomally or on plasmids. Up to now genes of *L. casei*, *Leu. lactis* and *Lactobacillus plantarum* were found to contain β -gal genes consisting of two overlapping reading frames (David *et al.*, 1992; Mayo *et al.*, 1994). It was the purpose of the present study to elucidate the location and the organization of the β -gal gene in *L. sake* and to compare it with known β -gal genes.

METHODS

Bacterial strains, growth conditions and plasmids. *L. sake* DSM 20017 and *L. curvatus* LTH 1432 were grown at 30 °C in MRS medium (de Man *et al.*, 1960) as described by Obst *et al.* (1992). *Escherichia coli* NM554 was used as the recipient for transformation and was propagated aerobically at 37 °C in SOB medium (Hanahan, 1985). Selective media contained 100 μ g ampicillin ml⁻¹. *E. coli* HB102 harbouring the *E. coli-Staphylococcus* shuttle vector pRB473 was kindly provided by R. Brückner, Tübingen, Germany. Plasmid pG⁺host5 was donated by E. Maguin, INRA, Jouy-en-Josas, France (Biswas *et al.*, 1993). The vector pBluescriptIIKS(+) was supplied by Stratagene.

Cloning procedures. Preparation of chromosomal DNA from *L. sake* DSM 20017 and Southern blot hybridization studies were described previously (Obst *et al.*, 1992). Genomic DNA of *L. sake* DSM 20017 was digested with *Hind*III. Fragments approximately 8–9 kb in size were isolated (DEAE cellulose, BioRad; Dretzen *et al.*, 1981) from 0.8% agarose gels and ligated into the de-phosphorylated *Hind*III-cleaved pRB473 vector with T4 DNA ligase (Boehringer). The ligation mixture was introduced into *E. coli* NM554 [Δ (lac17A); Bullock *et al.*, 1987] by transformation (Hanahan, 1985). *L. curvatus* was transformed by electroporation as described by Bringel & Hubert (1990). β -Gal-positive clones were detected as blue colonies on agar plates, treated with 50 μ l X-Gal solution (2%, w/v, in dimethylformamide) prior to use. *E. coli* plasmid DNA was obtained as described by Birnboim & Doly (1979). Subclones were constructed in pRB473 or pBluescriptIIKS(+) using standard techniques (Sambrook *et al.*, 1989).

DNA sequencing analysis. Double-stranded plasmid DNA was prepared by using the Qiagen plasmid purification method (Diagen). The nucleotide sequence was determined by the dideoxy chain-termination method of Sanger *et al.* (1977) employing the Sequenase (Tabor & Richardson, 1989) DNA sequencing kit (version 2.0, USB), [α -³⁵S]dATP (Amersham)

and primers (M13-20, Pharmacia; site-specific 17-mer primers, MWG-Biotech or Roth).

Computer analyses of sequences. The DNA sequences were analysed with the aid of a microcomputer system and DNAsis software (Pharmacia). Derived amino acid sequences were compared to the EMBL database using BESTFIT; for the alignment MULTALIGN was used (HUSAR).

RNA extraction. Cells of overnight cultures (10 ml) were harvested and resuspended in 250 μ l Tris/HCl (20 mM, pH 8.0). PEG 20000 (500 μ l, 24%, v/v) and 250 μ l Tris/HCl (20 mM, pH 8.0) containing 25 mg lysozyme were added and the mixture was incubated at 37 °C for 20 min. The suspension was centrifuged and the cells were lysed at 65 °C by adding 500 μ l pre-warmed sodium acetate (0.2 M, pH 4.8) containing EDTA (10 mM), 50 μ l 10% (w/v) SDS, and 500 μ l phenol. The mixture was adjusted to ambient temperature and 500 μ l chloroform were added. After centrifugation the aqueous phase was extracted once with phenol, twice with chloroform. RNA was precipitated at 4 °C by adding one volume LiCl (4 M).

Analyses of the mRNA. The length of the mRNA and the site of transcription initiation was determined in northern hybridizations and primer-extension analyses. Northern hybridizations were performed as described by Tichaczek *et al.* (1993) with a radiolabelled oligonucleotide probe (P1, 5'ATCTAACCATTGAATAT3') which hybridized close to the translational start codon. The hybridization temperature was 36 °C which was 6 °C below the *T_m* value calculated for 6 $\times$ SSC (Wetmur, 1991). For primer-extension experiments this and a second 17-mer oligonucleotide primer were used (P2, 5'CTGTTGTTGGCCATT3'). Their 3' ends were located 10 or 99 bases downstream from the translational start codon. RNA (20 μ g) was incubated at 37 °C for 1 h in 30 μ l reverse-transcription buffer (Gibco-BRL) containing 25 ng [γ -³²P]dATP-labelled primer, 0.5 mM of each dNTP, 50 mM DTT and 200 U M-MLV reverse transcriptase (Gibco-BRL). After ethanol precipitation, aliquots of the samples were electrophoretically analysed.

RESULTS

Cloning of an *L. sake* DNA fragment encoding β -gal

A digoxigenin-labelled 2.85 kb *Nco*I/*Hind*III fragment of pLZ15 inside the β -gal structural gene of *L. casei* ATCC393 (Flickinger *et al.*, 1986) was used to detect the location of the DNA sequence encoding the β -gal of *L.*

Fig. 1. Restriction map of pMOB1000 containing *lacL* and *lacM* of *L. sake* DSM 20017 and its deletion derivatives. The map of the inserts is depicted in pRB473 (pMOB1000–1500 and 3100) or pBluescriptIIKS(+) (pMOB3200). The polylinkers of pRB473 and pBluescriptIIKS(+) are indicated by a shaded box and a circle, respectively. The ability to complement the Lac⁻ phenotype of *E. coli* NM554 is indicated by + or –.

AGATTCTAACTTTAAATTAATGCTTTAAGGGTACTGGGAAAGTAAATATGATAACGATAAAAAATTAATCTGATAGTAAAGAACCCTGACACTAA	100
TTGTGTAGGGTTCTTTTGGACATCCAGCGGCAAACTTCTATACCTCAAAAAATGAACCGTTTAAATTTAAACTTGGAGGTTAAGTTTATGCAACCTAATATT	202
CAATGGTTAGATACACCGGCGCTTTCAGAGTGGGCCAATTCGCTGCCACAGTGATCACCGTTACTATGCGGACACTGGCCGAAATGGCCCAACACAGAGT	304
Q W L D T P A V F R V G L P A H S D H R Y Y A T L A E M A Q Q Q S	39
TCGTTTGAACAATCTTTGAATGGCAGGTGGCAATTTCACTACAGTGTAAATGCGGCGAGTCGTCTAAATCTTTTATGAATAGCATTTGATGCGCAAGAT	406
S F E Q S L N G T W Q F H Y S V N A A S R P K S F Y E L A F D A Q D	73
TTTGAACCAATCACAGTACCCCAACATATCGAATTTGGCGGGTTATGAGCAATTGCACTACATCAACACAATGTATCCGTGGGAAGGCGATTATATCGGCGT	508
F E P I T V P Q H I E L A G Y E Q L H Y I N T M Y P W E G H Y Y R R	107
CCCGCTTTTCAACGTCGATGCAAGCAACATCTCGCATGTTTAGTGAAGCGGATTAATCCCGTTGGTTCATACCTGCACCATTTTGATTTAAACGCCA	610
P A F S T S D D K Q H L G M F S E A D Y N P V G S Y L H H F D L T P	141
GCTTTGAGAAATCAACGGGTCATCATTCGCTTTGAAGGGTGAACAAGCGATGTATGTTGGCTCAATGGCCAAATTTATCGGCTATGCTGAAGATAGTTT	712
A L R N Q R V I I R F E G V E Q A M Y V W L N G Q F I G Y A E D S F	175
ACACCGTCTGAATTTGACTTAAACCCCTTATCTTAAAGAACTGATAATTCCTGGCGGTTGAAGTGCATAAGCGCAGTAGTCCGGCTTTTCATCGAAGATCAA	814
T P S E L T P Y L K E L D N C L A V E V H K R S S A A F I E D Q	209
GATTTCTTCGCTTCTTCGGGATTTTCGGGATGTTAAGTTATTAGCTAAACCCCGCACGCTTTGGAAGATTTATGGGTAATTCGGAATATACGCTGGTA	916
D F F R F F G I F R D V K L L A K P R T H L E D L W V I P E Y D V V	243
CAACAAACGGGACAGGTGAACCTTCGCTTCAATTTTCAGGAGATGAAACCGAGTCCATCTACGCATTTAGGACCAACATCAAATCATCTTAAACCGCGAT	1018
Q Q T G Q V K L R L Q F S G D E N R V H L R I R D Q H Q I I L T A D	277
TTAAGAGTGGCGCTCAGGTGAACGACCTTTATAAGATGCCGAATTTGGTTCAAGCGTGGTCAACCAACCCCTAACTTATACACATTTAGAGCTAGAAGTT	1120
L T S G A Q V N D L Y K M P E L V Q A W S N Q T P N L Y T L E L E V	311
GTGGATCAAGCTGGGGAACGATTGAAATTTAGTCAGCAACCAATTTGGCTTCGTAATAATGAGATTAAAGATAAAGTGATGTTGCTCAACGGAACCGGTTA	1222
V D Q F A G E I S Q Q P F G F I E I K D K V M L N G K R L	345
GTCATTAACGGCGTTAATCGGCATGAATGCATCTCGTGAACCTGGTGGGACAATACGCGCGAAGATGAAGCGTGGGATATTGCTTGCATGCGCAACCAAT	1324
V I N G V N R H E W H P E T G R T I T A E D E A W D I A C M Q R N H	379
ATCAATGCGTCCGAACATCGCATTATCCAGATCGGTTATCTTTTATAATGGTTCGCATCAAGCTGGGCATCTATATGATGGCTGAAACCAATTTGGAATCG	1426
I N A V R T S F Y P D R L S F Y N G C D Q A G I Y M M A E N L E S	413
CATGTTCTTGGCAAAAAATGGGGCGGTGCAACCTTCTTGAACGTCGCCAGGTTTCATATGATGAATGGGAAGCGGCGACGCTCGATCGAACCAGCAACCAAT	1528
H G S W Q K M G A V E P S W N V P G S Y D E W E A A T L D R A R T N	447
TTTGAACGTTTAAAAATCACGCTCTCAATTTCTTTTGGTCATTAGGGAATGAATCTTACGCTGGTAGTGTCTTTGAGAAGATGAACGCCCTATTACAAGCAA	1630
F E A T G F T S I L F N H S L G N S Y A G S V L E K M N L E S	481
CAAGTCCAAACGCTTAGTGCAATTATGAAGGGTCTTCGGGCGCCGAGTATAAGGCGACGATTTCGGATGTTGAAAGTCGGATGTATGCGACACCGGCT	1732
Q D P T R L V H Y E G V F R A P E Y K A T I S D V E S R M Y A T P A	515
GAGATTAAAGCTTATTAGATTAACGCCCCACAAAACCAATTTATCTCTCGCAATATATGATGATATGGGAATTTGCTGGGTGGTATGCAATCGTATATC	1834
E I K A Y L D N A P Q K P F I L C E Y M H D M G N S L G G M Q S Y I	549
GACTTATTGTCACAATATGACATGTACCAAGGCGGCTTTATCTGGGATTTTCATGATCAAGCACTCTTGGTGACAGATCCAGTGACTGGTCAACGCTGAATTA	1936
D L L S Q Y D M T Q G G F I W D F I D Q A L L V T D P V T L E S	583
CGCTACGCTGGCGATTTTGTATGACCGCCCATCAGACTATGAATTTTCGGGGGATGGGTTAGTCTTTGCTACCCGCGATGAAAAACAGCAATGCAAGAGGTT	2038
R Y G G D F D D R P S D Y E F S G D G L V F A T R D E K P A M Q E V	617
AGATACTATTATGCGCAACACAAATAAACGCTTAGCAGTGATCTTCGGAGATGTGACTTTAGGACTTAAAGGCCCTGACTTTCACTACCTCTTTTCGTACCA	2140
R Y Y Y G E H K *	625
M A N T N K R L A V I F G D V T L G L K G P D F H Y L F S Y Q	31
AACGGGTGGGCCAGATCATTCGCGGATTCAGGTAAGGAATGGCTATATCGCTCACCACCAACCGACTTTTGGGGGGCAATACCGATAATGATCGCGGCA	2242
T G G F S E L R I Q G K E W L Y R S P K P T F W R A T T D N R G N	65
CCAATTTCCCATTTGAATTCGGAATGGCTAGCTGCTGATCAGTTTATGCTTGTGAGTCGATTACAGTGGCGATTGACGGGCAACGATTTTCGCTGCCAAT	2344
Q F P L K S G M W L A A D Q F I A C Q S I T V A I D G Q T I P L P I	99
TGCGCCGAGAACACCGCTATAGTGGTGGGAAACGGCCCAAGAGTGACGGTTACGTTATACCTATCAACAAATTACGACGCGCAGACGCGTAGAAGT	2446
A P E N N R Y S G R E T A Q E V T V T Y T Y Q T I T T P Q T T V E V	133
CAGTTATACGATTCAAGCCAGTGAATAATCCGTAGCGGTGACTTATCATGGGCAAGCTGGATTACCATCATTTGCGAGCTTTTGGCTTACGCTTTGTCTA	2548
S I Q A T I R V A S G K I R V T Y H G Q A G L P S L P V F V M	167
GCCGACCCACGCGCGCTTTATTATCAAGGGTTGTCAGGGGAGACTTATCCAGATCGAATGGCTGGTGGGATTGCTGGGGAATACGAAGTACACGGGCT	2650
P T P A T R F I Y Q G L S G E T Y P D R M A G G I A G E Y E V T G L	201
ACCTGTGAGCGCTTATCTAGTGCCACAAGATTGTGGTGTGCATATGCAACCGATTGGGTGACGATTATCGGCAAGCGGTGCTGGATAATTTGTGACGA	2752
P V T P Y L V P Q D C G V H M A T D W V T I Y R Q A V L D N C L H E	235
ACCAGTCGAACCGGCTTGAAGTTTAAAGTGGTCGATCAACCATTTGCTTTTCATGTTTCCATATACCGCTGAAGAATTGGAATAATCGACCCATCATTC	2854
P V E T G L K F K M V D Q P F A F S C L P Y T A E E L E N A T H H S	269
AGAECTACCCGACCAACCGACAGTCTTAAATCTCTTAGGGGCGGTCGTGGCGTGGGCGGTATCGGATAGTTGGGGTAGCGAGCTAGAAGTGGCCCTATCA	2956
E L P A P V L N L G A V R G V G G I D S W G S D V E A V T Q	303
AATAGATGCGACGAGGATCGCCATTTTGAATTTGAAATTTTCGTTTAAAAAGTAAAAAGAGTTGCGGCAAAAAATCATTTTTCGCGCAACTCTTTTATTTG	3058
I D A T Q D R H F E F E I S F *	318
TAAAGGGCTGTGGCGGCGCTCTGTTTTCAAATCAGCTATAATAGTAAATAATATGTTTATTTGGTGGGCAATTCGCTGGGAAATAATGGTGAATCGAGGT	3160
AATGATAATGGAAGCTGAACAAATGATAATTTTAAACCGCTAGAGACTGTTTATAGATTTAGGGAAGCTATTGTTCAAGCGCATTTGAAGGTGCC	3262
ACTTACCTTGGATCGCGAGCAATCACAAGAACTGTTCGACAAAAATAATCGTTTTCG	3318

Fig. 2. Nucleotide sequence of *lacI* and *lacM* encoding the β -gal of *L. sake* DSM 20017. Important features in the sequence are underlined and marked: -10 and -35, promoter region; 1 and 1', sites of transcription initiation determined with mRNA from *E. coli* NM554(pMOB1000) and mRNA from *L. sake* DSM 20017, respectively (compare Fig. 3); rbs, ribosome-binding sites; *lacI* and *lacM*, start of the ORFs; t, terminator; and relevant restriction sites.

sake DSM 20017 (Obst *et al.*, 1992). A signal was detected with an 8.65 kb *Hind*III fragment of chromosomal origin. A gene library of *L. sake* DSM 20017 was constructed in pRB473, using *Hind*III fragments the same size as the hybridizing ones. The β -gal-negative phenotype of *E. coli* NM554 was complemented in 2.5% of the transformants. The recombinant plasmids were stably maintained in *E. coli*. Plasmids of 10 β -gal-positive clones contained an 8.65 kbp *Hind*III fragment of chromosomal *L. sake* DNA. This was confirmed by re-hybridization of digested and undigested genomic *L. sake* DNA with probe consisting of a 1.1 kbp *Acc*I/*Sph*I fragment inside the cloned *Hind*III fragment. Both orientations of the insert were obtained equally and the respective plasmids were designated pMOB1000 and pMOB3000. The restriction map of the insert in pMOB1000 is depicted in Fig. 1.

Construction of deletion clones

To localize the DNA sequences on the 8.65 kb *Hind*III fragment which are necessary to express an active β -gal in *E. coli*, pMOB1000 or pMOB3000 were digested with restriction enzymes, re-ligated and introduced into *E. coli* NM554. In Fig. 1 an overview is presented of the constructed plasmids and the corresponding phenotype of the transformants. Plasmid pMOB1500, carrying a 4.15 kb *Pvu*II/*Hind*III insert, was the smallest plasmid which induced blue colonies on agar containing X-Gal.

Nucleotide sequence analysis

The DNA sequence of a 3.3 kb region of pMOB1000 was determined and is shown in Fig. 2. The coding region for the β -gal of *L. sake* is located at nucleotides 188–3005 and consists of two ORFs on the same strand which are overlapping in an area of 17 nucleotides. In analogy to the β -gal genes of *Leu. lactis* (David *et al.*, 1992) the ORFs were denoted *lacL* and *lacM*, respectively (Fig. 1). *lacL* is comprised of 1878 bp, encoding 625 amino acid residues with a calculated molecular mass of 72 453 Da. The second ORF, *lacM*, is located downstream of *lacL* and comprises 975 bp, encoding 318 amino acid residues with a molecular mass of 35 252 Da. A terminator sequence consisting of a palindrome with a calculated free energy of $-24.8 \text{ kcal mol}^{-1}$ ($104.2 \text{ kJ mol}^{-1}$) was present only downstream of *lacM*, indicating a rho-independent transcription termination. Putative RBSs were identified upstream of both *lacL* and *lacM*.

Analysis of the nucleotide sequence upstream of *lacL* revealed the presence of a putative coding region, 510 bp of which were sequenced. The deduced amino acid sequence of this region did not share similarities with known proteins of the lactose metabolism, transposases or other proteins.

mRNA analyses

Northern blot hybridizations revealed a weak signal indicating the formation of a single transcript of approximately 2.9 kbp with mRNA of *E. coli*

Fig. 3. Determination of the sites of transcription initiation in the *lacL* gene. The autoradiogram depicted is of a primer-extension experiment with primer P1 using RNA from *L. sake* DSM 20017 (lane 1) and *E. coli* NM554(pMOB1000) (lane 6). Samples of a sequencing experiment using the same primer were analysed on lanes A (ddATP), C (ddCTP), G (ddGTP) and T (ddTTP). Transcriptional start sites are indicated by arrows and are located 34 and 106 bp upstream of the start codon of *lacL* (compare Fig. 2). The same signals were obtained in an independent experiment using P2 as primer (data not shown).

NM554(pMOB1000) and (pMOB1400). Thus, both ORFs were transcribed as a single mRNA. The result of the primer-extension analysis with primer P1 is depicted in Fig. 3. Within mRNA isolated from *E. coli* NM554(pMOB1000) and *L. sake* DSM 20017 the site of transcription initiation was identified as an adenine residue 34 bp upstream of the *lacL* translational start codon. Sequences similar to the consensus sequence of the *E. coli* promoter (Hawley & McClure, 1983) were detected in the -10 and -35 regions upstream of the transcription initiation site. No such sequences were identified upstream of the *lacM* translational start codon. With mRNA of *L. sake* DSM 20017 an additional signal was obtained in primer-extension analysis located 106 bp upstream of the *lacL* start codon. However, no similarities to known *E. coli* promoter consensus sequences were identified in the respective -10 and -35 regions. Primer-extension analysis with primer P2 revealed the same initiation signals, but a different unspecific background. The promoter sequence is indicated in Fig. 2.

Phenotypic expression of the β -gal

For the expression of the β -gal of *L. sake* DSM 20017 in the β -gal-negative strain *L. curvatus* LTH 1432, the region containing *lacL* and *lacM* was subcloned in pG⁺host5. Therefore a 5.4 kbp *Hind*III/*Pst*I fragment was ligated into the multiple cloning site of pG⁺host5. The resulting plasmid, designated pGB100, was transformed in *L. curvatus* by electroporation. The expression of a functional β -gal in clones containing pGB100 was indicated by the formation of blue colonies on MRS agar containing X-Gal.

DISCUSSION

The β -gal of *L. sake* DSM 20017 is encoded by two overlapping genes. Plasmid variants lacking parts of *lacL* or *lacM* were unable to produce an active β -gal, indicating that both are required for formation of the functional enzyme. The same was found for *lacL* and *lacM* of *Leu. lactis* which also share a 17 bp overlap (David *et al.*, 1992). No other ORFs were detected adjacent to the genes of *Leu. lactis*. In contrast, the *lacZ* gene encoding the β -gal of *L. bulgaricus* is organized in an operon structure together with the lactose permease gene which is located upstream of *lacZ*, separated by only 3 bp (Leong-Morgenthaler *et al.*, 1991). In *L. sake* an ORF of hitherto unknown function was detected upstream of *lacL*. No similarities to other proteins were detected after comparison with the partially determined sequence of this region. However, it seems unlikely that an operon-like organization of lactose genes is present in *L. sake* since the promoter and terminator sequences are present only upstream and downstream of *lacL* and *lacM*, respectively, and only a single transcript of 2.9 kbp is formed.

In two independent primer-extension experiments using different primers and mRNA of *L. sake* DSM 20017, two signals, only one of which was preceded by an *E. coli* promoter consensus sequence, were obtained. Thus, it cannot be excluded that *L. sake* uses other promoter signals in addition to the known -10 and -35 consensus sequences. Upstream of both, *lacL* and *lacM* putative RBSs were detected with the sequences 3'GGAGG5' (*lacL*) and 3'AGGAGG5' (*lacM*). These sequences are complementary to the 3' end of the 16S rRNA of the known consensus sequence found in many organisms. Primers based on the 3' end of the 16S rRNA sequence of *L. sake* (W. Ludwig, Technische Universität München, Germany, personal communication) gave the expected amplified product when used in a PCR reaction with *L. sake* DNA. This indicates the presence of a sequence in the *L. sake* 16S rRNA which is complementary to the RBSs detected for *lacL* and *lacM*. This finding is different from the absence of an effective RBS upstream of *lacM* of *Leu. lactis* (David *et al.*, 1992). However, translational coupling of the *L. sake lacL* and *lacM* genes cannot be excluded because of their overlapping sequences. This organization would ensure production of equal amounts of *lacL* and *lacM* expression products forming a heterodimer as suggested for *Leu. lactis* (David *et al.*, 1992).

lacL and *lacM* are located on the chromosome of *L. sake*

LacL	
MQPNIQWLDTPAVFRVQQLPAHSDHRYATLAEMAQOQSSFEQSLNGTWQFHYSVNAASRPKSFYELAFDAQDFE	75

MQANLQWLDDPEVFRVQQLPAHSDHRYATLAEMAQOQSSFEQSLNGTWQFHYSVNAASRPKSFYELAFDAQDFE	74

PITVQHIELAGYEQQLHYINTMYPMEGHYRRPAPFSTDDKQHLQMPSEADYNPVGSYLHFDLTPALRNQRII	150

TIQVGHIELAGYEQQLHYINTMYPMEGHYRRPAPFSTDDKQHLQMPSEADYNPVGSYLHFDLTPALRNQRII	149

RFBGEQAMVYVNLQGFYAGDSPTTSEFDLTPYLKETDNCLAVEHKKSSAIFEDQDFRFFGIFRDKVLLA	225

QFGQVEEALYVNLQGFYAGDSPTTSEFDLTPYLKETDNCLAVEHKKSSAIFEDQDFRFFGIFRDKVLLA	224

KPRTHLEDLWVPEYDVVQQTQVKRLQFSGDENRHLRIRDOHQIILTADLTSG-AQVNDLYKMPVQAWSN	299

EPASHITDLDIRPVNANLKSGLNITTKVTGEPATLALTVKHGDRVLTSTQQTGSGSVTFDTNLDQLHLMS	299

QTPNLYTLELVVDQAGETIEISQPPGFRKIEIKDKVNL-LNQLRVLVGNVRHSHHPETGRTITAEADNDIA	373

QTPYLYQLTIEVVDADHQLLEVVPYQGFRTVELRDKVIYVNNKRVLVGNVRHSHHPETGRTITAEADNDIA	374

CMQRHINAVRTSHYPDRLSFYNGCDQAGIYMAETNLSHGSGWQKGAIVEPSWNVPGSYDEWEAATLDRARTNF	448

TMLANNINADRTCHYPQQLPMYQLCDEAGIYMAETNLSHGSGWQKGAIVEPSWNVPGSYDEWEAATLDRARTNF	449

ETFKNHISILFWSLGNESYAGSVLEKQNAIYKQDPTLHVHYEGVFRAPPEYKATISDVESRMATPAEIKAYLDN	523

EWFKNHPSIIFWSLGNESYAGSDIAAMQAFKEHDSRLVRYEGVFTPELKRISDVESRMATPAEIKAYLDN	524

APQKPFILCEYMDMNSLGGMSYIDLLSQYDMYGGFIWDFIDQALVTDPTVGTQRELRYGGDFDRPSDYEF	598

NPTKPFILCEYMDMNSLGGMSYIDLLSQYDMYGGFIWDFIDQALVTDPTVGTQRELRYGGDFDRPSDYEF	599

SGDGLVFPATREKPMQEVRYTYGEHK	625

SGNLMFADRTPKPMQEVRYTYGLHK	626
LacM	
MANTNKLAVIFGDVTGLKGPDPHYLFSYQGGPESLRIQKELVYRSPKPTFRATTNDNRGNQFLKSGMWL	75

MAYTNQLHIVYGDGSLGQGANFHYLFSYERGGLESVVNDKELWLYRTPPMFWRATTNDNRGSGFSGSAQWY	75

AADQFIACQSITVAIDGQ-TIPLIAPENNRYSGRETAQEVTVTYTQITTPOTTVEVSYTIQASGRKIRVAVTY	149

AADKFTSCQDLELTVDDQVTPPLIAPLNNKYTDHEIATKVSILAVHGVTTTVPSTIVTVTYTADQGINIATHY	150

HGQAGLPSLPVFLGRFVMPPTATRFIYQGLSGETYPRDMAGGAGEVETGLPVTPLVPQDCGVHMDMTVYI	224

SGQSDLPFLPAFLGRFIMPPTATGFDYTGLSGETYPRDLGATHGQHFVDSLPVTPLVPQDCGVHMDMTVYI	225

RQAVLDNCLHEPVTGLKFMVDQPAFASCLPYTAELNATHHSELPAHRTVLNLLGAVRGVGGIDSWGSDVE	299

RSTTQNNADHNTFSLTFSQTDAPPAFASCLPYTAELNATHHSELPAHRTVLNLLGAVRGVGGIDSWGSDVE	300

VAYQIDATQDRHFEFEISF	318

APYHILANDIDFSPNTHF	319

Fig. 4. Amino acid sequence alignment of LacI and LacM of *L. sake* (top line) and *Leu. lactis* (bottom line). Both proteins are required for the formation of a functional β -gal. Identical amino acids are indicated by asterisks.

and thus differ from the plasmid-encoded genes of *L. casei* ATCC 393 (Flickinger *et al.*, 1986), *Leu. lactis* NZ 6009 (David *et al.*, 1992) and *L. plantarum* (Mayo *et al.*, 1994). Whereas the β -gal genes of *Leu. lactis* and *L. casei* share an almost identical nucleotide sequence of 99%, the corresponding value for the β -gal genes of *L. sake* was only 65.3%. Only three segments of the sequence of the *L. plantarum* gene were published, thus not allowing proper comparison. The sequence of this segment, however, was very similar (98%) to the *Leu. lactis* and *L. casei* genes (Mayo *et al.*, 1994). The difference in the *L. sake* genes is consistent with a different codon usage as *L. sake* prefers A- and T-rich codons (data not shown). This was also observed with other genes of *L. sake* encoding the catalase (Knauf *et al.*, 1992) and sakacin P (Tichaczek *et al.*, 1994). On the other hand, the dissimilarity of the chromosomal *lacL* and *lacM* genes of *L. sake* from those of *L. casei* and *Leu. lactis* encoded on plasmids may be the result either of spontaneous deletion and insertion events or equally distributed changes in the amino acid sequence causing divergence of genes from a common ancestor. Spontaneous deletions were observed within the β -gal gene of

Table 1. Homology between *L. sake* DSM 20017 β -gal with those of other organisms

Species (reference)	No. of amino acids	Identical amino acids (%)	Similar amino acids (%)
<i>Lactobacillus sake</i> DSM 20017 (this study)	625 + 318	100	100
<i>Leuconostoc lactis</i> * (David <i>et al.</i> , 1992)	626 + 319	59.1	72.3
<i>Streptococcus thermophilus</i> (Schroeder <i>et al.</i> , 1991)	1026	44.0	63.0
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> (Schmidt <i>et al.</i> , 1989)	1006	42.2	60.2
<i>Clostridium acetobutylicum</i> (Hancock <i>et al.</i> , 1991)	897	48.0	64.8
<i>Clostridium thermosulfurogenes</i> (Burchhardt & Bahl, 1991)	716	26.8	51.6
<i>Bacillus stearothermophilus</i> (Hirata <i>et al.</i> , 1986)	672	16.8	42.4
<i>Escherichia coli</i> (Kalnins <i>et al.</i> , 1983)	1023	31.8	54.0
<i>Klebsiella pneumoniae</i> (Buvinger & Riley, 1985)	1034	32.3	54.0
<i>Homo sapiens</i> (Oshima <i>et al.</i> , 1988)	677	17.4	44.1

* The *Leu. lactis* β -gal shares more than 99% homology with the *L. casei* enzyme. Partial sequences of the β -gal gene of *L. plantarum* are also very similar to the *Leu. lactis* gene (Mayo *et al.*, 1994).

L. bulgaricus NCDO 1489 (Mollet & Delley, 1990), and ISL3 was described as a new IS element responsible for such deletions (Germond *et al.*, 1993). For *L. sake*, the presence of 29 direct and 25 inverted repeats of 9 bp or more in *lacL* and *lacM* and the observation of homologous silent genes in strains other than DSM 20017 might suggest similar mechanisms (Obst *et al.*, 1992). However, the alignment of the sequences from *Leu. lactis* and *L. sake* depicted in Fig. 4 shows equal distribution of conserved amino acids. At different positions one amino acid was inserted (3 $\times$) or deleted (1 $\times$) in the *Leu. lactis* β -gal. Therefore, the difference in the β -gal of *L. sake* should not be a result of deletion or insertion. The similar plasmid-encoded β -gals were probably acquired more recently from a common source than the chromosomally encoded β -gal of *L. sake* which has diverged.

The *L. sake* and *Leu. lactis* β -gals share 59.1% identity. An overview of the homologies of the *L. sake* β -gal to other β -gals is provided in Table 1. Amino acids which are described to be involved in the formation of the catalytic centre in *E. coli* are conserved (Herrchen & Legler, 1984). Nevertheless, the β -gal activity of *L. sake* DSM 20017 was low compared to *L. casei* (Obst *et al.*, 1992). This may be caused by the relative dissimilarity to the *L. casei* and other β -gals or a less effective promoter. The latter is supported by the weak signals obtained in Northern hybridization and primer-extension experiments.

The organization of the β -gal as a heterodimer as described for *Leu. lactis* (David *et al.*, 1992) is very likely and thus, an α -complementation system for self-cloning in *L. sake* could be developed as described for *E. coli* and *Bacillus subtilis* (Zabin, 1982; Haima *et al.*, 1990). Furthermore, a

functional β -gal is expressed in *L. curvatus*, all strains of which were found to be deficient in β -gal (Obst *et al.*, 1992). Hence, the genes encoding the β -gal could replace antibiotic reporter genes in food-grade vector systems for meat lactobacilli.

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