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Molecular characterization of the α-amylase genes of *Lactobacillus plantarum* A6 and *Lactobacillus amylovorus* reveals an unusual 3′ end structure with direct tandem repeats and suggests a common evolutionary origin

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Abstract

The α-amylase gene (amy*A) of Lactobacillus plantarum* A6 was isolated from the genome by polymerase chain reaction with degenerated oligonucleotides, synthesized according to the tryptic peptide amino acid sequences of the purified enzyme. Nucleic acid sequence analysis revealed one open reading frame of 2739 bp encoding a 913 amino acid protein. The amylase appears to be divided into two equal parts. The N-terminal part has the typical characteristics of the well-known α-amylase family (65% identity with the α-amylase of *Bacillus subtilis* and 97% identity with the partial sequence available for the α-amylase of *Lactobacillus amylovorus*). The C-terminal part displays a fairly unusual structure. It consists of four direct tandem repeated sequences of 104 amino acids sharing 100% similarity. The complete nucleotide sequence of the α-amylase gene of *L. amylovorus* was also determined. An open reading frame of 2862 bp encoding a 954 amino acid protein was identified. Perfect homology between the two *amy*A genes was observed in the N-terminal region. The C-terminal part of *L. amylovorus* α-amylase also included tandem repeat units but striking differences were observed: (i) the addition of one repeat unit; (ii) a shorter, 91 amino acid repetition unit. These structural homologies suggest that both genes have a common ancestor and may have evolved independently by duplication with subsequent recombination and mutation. © 1997 Elsevier Science B.V.

Keywords: Lactic acid bacteria; Gene organization; Repeated sequences; Evolutionary origin

1. Introduction

Lactobacillus plantarum is recommended as starter culture for fermentation controls of silage, cabbage, cucumber, olive and cassava (Vaughn, 1985; Seale, 1986; Daeschel and Fleming, 1987). Silage sometimes contains too little fermentable sugar to ensure the rapid production of a stabilizing amount of lactic acid. To overcome this difficulty, attempts have been made in recent years to produce a recombined strain of *L. plantarum* releasing a high amylolytic activity by the introduction of the α-amylase gene of *Bacillus amyloliquefaciens* (Jones and Warner, 1990) or *Bacillus stearothermophilus* (Scheirlinck et al., 1989). Nevertheless, the amount of α-amylase excreted by such recombinants was negligible. Recently, Fitzsimons et al. (1994) developed an amylolytic *L. plantarum* strain by the insertion in the chromosome of an active fragment of the gene encoding α-amylase production in *L. amylovorus*. The recombined *L. plantarum* strain was able to achieve amylolytic activity at a level similar to that of *L. amylovorus*. However, the use of such a genetically engineered strain under natural fermentation conditions may raise legal or ecological problems.

A wild strain of *L. plantarum* (strain A6) was previously isolated from retted cassava and selected for its ability to break down soluble starch (Giraud et al., 1991). The characteristics of this strain are particularly attractive. An amylolytic *L. plantarum* strain was reported for the first time. Moreover, it was found to

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synthesize large amounts of α-amylase. It was recently shown that *L. plantarum* A6 can also break down cassava raw starch without any preliminary physico-chemical treatment (Giraud et al., 1994). New applications of *L. plantarum* A6 might result from these promising results. It could be used as a starter when traditional fermentation processes of starch-containing food fail to produce sufficient lactic acid.

Molecular study of the amylase gene (*amy*A) of *L. plantarum* A6 was used to follow the development and the dispersion of the microorganism during traditional fermentation processes and to understand the origin of its unusual property.

Many microbial and eukaryotic α-amylase genes have been cloned and characterized (Horii et al., 1987; Korman et al., 1990; Sutliff et al., 1991; Rumbak et al., 1991). However, little work has been performed on the α-amylase genes of lactic acid bacteria. Only the α-amylase gene of *Streptococcus bovis* has been completely sequenced (Cotta and Whitehead, 1993), and the nucleotide sequence of the 5′ end of the *L. amylovorus* amylase gene has been determined (Fitzsimons et al., 1994). No significant homology has been found between these two genes.

The cloning and sequencing of the α-amylase gene of *L. plantarum* A6 are described here and the unusual structure of the 3′ end of this gene is shown. Furthermore, as substantial homology was observed with the 5′ end of the *L. amylovorus* amylase gene (>97%), the latter was fully characterized to determine the extent of the homology.

2. Materials and methods

2.1. Bacterial strains and growth media

L. plantarum A6 was isolated from retted cassava (Giraud et al., 1991) and *L. amylovorus* CIP 102989 was purchased from Institut Pasteur (France). For cloning experiments, *Escherichia coli* XL1-blue was used as a host strain. MRS (Difco Laboratories, Detroit, MI, USA) and LB (GIBCO) media were used for routine culturing of lactobacilli and *E. coli*, respectively.

2.2. Purification of the α-amylase and amino acid sequencing

α-Amylase of *L. plantarum* A6 was purified as described previously (Giraud et al., 1993). An aliquot of the protein (approx. 300 pmoles) was treated with 0.5 μg trypsin in 0.1 M Tris buffer (pH 8.1) overnight at 37°C. The resulting digest was fractionated by reverse-phase HPLC on a Brownlee C_8, 2 × 100 mm column (Perkin Elmer, Foster City, CA, USA) and eluted by a linear acetonitrile gradient in 0.1% trifluoracetic acid. The eluate was monitored by UV densitometry (220 nm). The resulting peptides were further purified using a Brownlee C_{18} column with the same experimental conditions. Selected peptides were submitted to Edman N-terminal sequential analysis in a 470-A protein sequencer connected on-line with a 120-A PTH analyser (Perkin Elmer). Both were operated according to the manufacturers' recommendations.

2.3. Southern hybridization analyses

L. plantarum A6 genomic DNA prepared as described by Gasson and Davies (1980) was digested with restriction endonucleases, separated by agarose gel electrophoresis and transferred to nylon membranes (Hybond-N^+; Amersham, Amersham, UK) using standard methods (Sambrook et al., 1989). DNA probes were labelled with digoxigenin (DIG)-dUTP using the DIG labelling kit supplied by Boehringer-Mannheim (Mannheim, Germany). Hybridization and detection experiments were performed with a DIG detection kit (Boehringer-Mannheim).

2.4. Amylase gene cloning

Total DNA was digested to completion by *Hin*pI and size-fractionated on 1% agarose gel. The 2.7 kb fragments bearing the α-amylase gene were eluted from the gel and ligated into the *Acc*I site of plasmid pGEM-3Zf(+) (Promega, Madison, WI, USA). Transformation of *E. coli* electro-competent cells was performed with a Bio-Rad Gene Pulser unit (Bio-Rad, Hercules, CA, USA). After replica plating, the positive clones containing the *amy*A gene were screened from the gene libraries by colony hybridization under the same conditions as those used for southern hybridization analyses.

2.5. DNA amplification and cloning of PCR fragments

PCR reactions were performed in 50 μl final volume containing: 0.1–0.5 μg of DNA and deoxyribonucleoside triphosphate (200 μM each), primers (0.8 μM each), $MgCl_2$ (1.5 mM), 1.25 unit *Taq* DNA polymerase (Promega) and the buffer supplied with the enzyme. The amplification conditions were: initial denaturation at 94°C for 5 min, 30 cycles with a 30 s denaturation step at 94°C, 30 s annealing step at 50 or 55°C (according to the T_m of primers used) and an elongation step at 72°C lasting 1–3 min (depending on the length of fragment to be generated). After the final elongation step at 72°C for 7 min, the amplified fragments were purified by a Wizard procedure and ligated into pGEM-T vector (Promega).

2.6. Sequencing of the DNA

Both strands of the inserts of the different clones were sequenced by the dideoxy-chain termination method (Sanger et al., 1977) with an 373 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Protein homology searches were carried out with the SWISS-PROT (release 34) database and genomic sequences with the GenBank (release 98) database and the FASTA program (Pearson and Lipman, 1988).

2.7. Nucleotide sequence accession numbers

The GenBank accession numbers for *L. plantarum* A6 and *L. amylovorus* α-amylase genes are U62095 and U62096, respectively.

3. Results

3.1. Amplification of a specific probe of α-amylase gene of L. plantarum

Purified α-amylase of *L. plantarum* A6 (Giraud et al., 1993) was subjected to trypsin digestion and the resulting peptides were separated by reversed-phase chromatography. Three peptides were selected for sequencing analysis, and the sequences (Table 1) were compared with those of the protein data bank (SWISS-PROT). Strong homology was found for the three peptides with the sequence of the α-amylase of *Bacillus subtilis* (Yang et al., 1983). The degenerate primers A and B were then designed from peptide 1 (amino acids DWTHGNT) and peptide 2 (amino acids WPNITDN). The marked homology found with the α-amylase of *B. subtilis* led us to define primer C (amino acids EFKEMCA) corresponding to the less degenerated amino acid region of the AMYA of *B. subtilis*.

Primers A–B led to the amplification of a 253-bp fragment and primers B–C to the amplification of a 371-bp fragment from the genomic DNA of *L. plantarum* A6. Their sequences revealed a high identity level (65%) with the α-amylase gene of *B. subtilis*.

3.2. Isolation of the L. plantarum A6 amyA gene

Southern hybridization analyses with the 371-bp DIG-labelled fragment as a probe was performed (Fig. 1). Two bands were observed, except for *Pst*I digestion. The variation range (600–700 bp) between the two bands was similar for all the digestions. Since the enzyme sites used in this analysis were not present in the probe (except for *Hin*pI, whose site is located at the 5′ end of the probe) and since no hybridization signal was observed with the plasmidic fraction of *L. plantarum* A6 DNA (data not shown), these results suggest that *L. plantarum* A6 contains at least two chromosomal *amy*A gene copies.

A first attempt was made at cloning the entire gene. The 5.2-kb *Hin*dIII fragments were eluted from an agarose gel and ligated into the dephosphorylated *Hin*dIII site of vector pGEM-3Zf(+). No hybridization signal was observed after screening 1200 recombinant clones. These results suggest that the entire amylase gene or another region of the chromosome flanking the amylase gene was lethal in *E. coli*, as has already been observed for *B. subtilis amyA* gene cloning (Yang et al., 1983; Emori et al., 1990).

It was therefore decided to isolate the gene in truncated segments. The 2.7-kb *Hin*pI fragment pool was isolated from the gel, and cloned into the vector pGEM-3Zf(+) linearized with *Acc*I. Of the 500 recombinant clones, five hybridized with the 371-bp DIG-labelled fragment, one of these was then sequenced.

The 2.7-kb fragment corresponds to approximately 80% of the DNA that encodes the mature part of the α-amylase protein but the 5′ end of the entire gene is lacking. This 5′ end was then obtained using a PCR screening plasmid approach. The 5.2-kb *Hin*dIII fragment pool was isolated from a gel and ligated into a pGEM-3Zf(+) vector linearized with *Hin*dIII. A primer deduced from the *Hin*pI fragment sequence and the universal oligonucleotides (M13 forward and M13 reverse) of the vector plasmid were used to amplified a 1 kb fragment corresponding to the lacking region of the *amy*A gene.

Table 1
Peptide sequences of the α-amylase of *L. plantarum* A6 and their homology with the α-amylase of *B. subtilis*

Peptide No.	Sequence	Homology with AMYA of *B.subtilis* (amino acid location)
1	SISDWTHGNTQISNWSDR	SISD WTHGNTQISN WSDR II:: IIIIIIII:I IIII 151 ISNEVKSIPN WTHGNTQIKN WSDRWDVTQN 181
2	HIELPSQYDGSYGSNFWPNITDNG	HIELPTQYDG SYGSNFWPNI TDNG IIIII: II IIII:IIIII I::: 221 HIELPD--DG SYGSQFWPNI TNTSAEFQYGEI 251
3	LGWAVVASR	LG WAVVASR II III:III 321 WMSDDDIRLG WAVIASRSGS 341

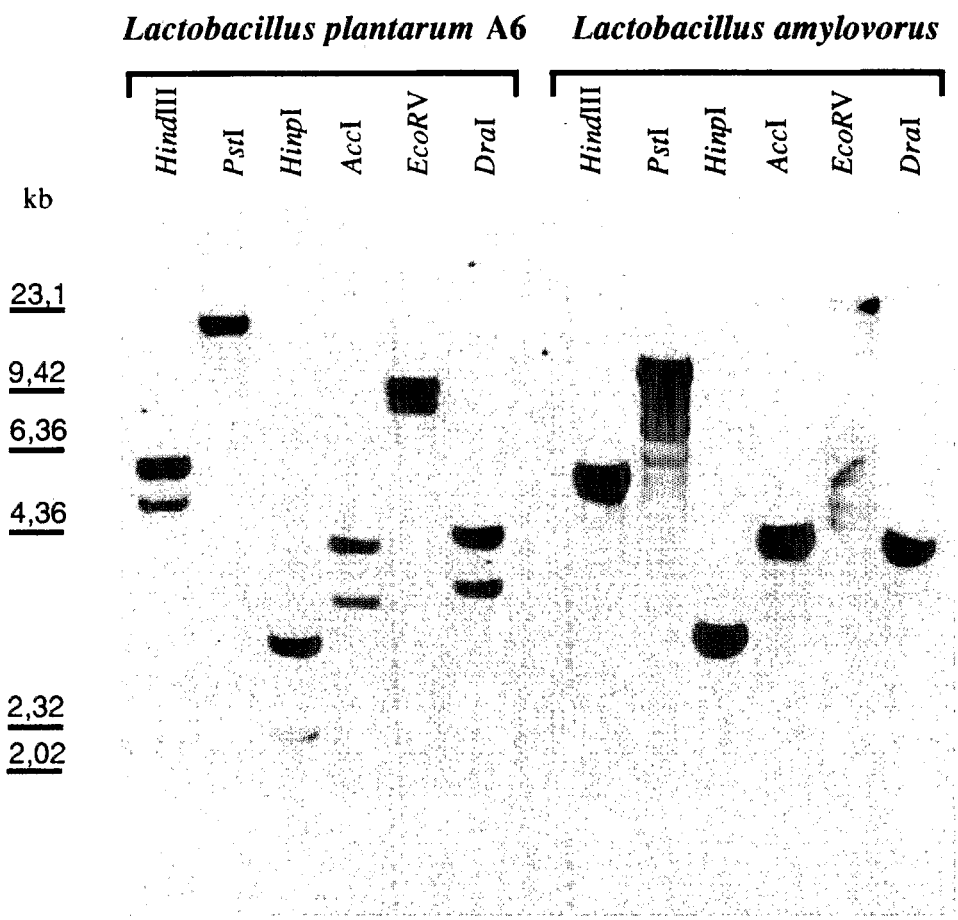

Fig. 1. Southern hybridization analyses of the α-amylase genes of *L. plantarum* A6 and *L. amylovorus*. Chromosomal DNA of *L. plantarum* A6 and *L. amylovorus* digested with *Hin*dIII, *Pst*I, *Hin*pI, *Acc*I, *Eco*RV and *Dra*I was subjected to electrophoresis on 1% agarose gel and blotted on nylon membranes. The 371-bp DIG-labelled DNA (fragment B–C) was used as a probe.

3.3. *Nucleotide sequence of L. plantarum A6* amy*A gene*

An open reading frame (*amy*A) of 2739 bp encoding a 913 amino acid protein with a molecular mass of 99 544 Da was identified. Fig. 2 shows that the open reading frame starts with a GTG codon at nucleotide position 363, which is preceded at a canonical distance (9 nucleotides) by a putative ribosome-binding site (AAAGGGGG) complementary to the 3′ end of the *L. plantarum* 16S rRNA (UCUUUCCUCCAC; complementary bases underlined) (Woese et al., 1992, unpublished).

The first 108 nucleotides of the structural gene encode a characteristic Gram-positive signal peptide of 36 amino acids (von Heijne, 1985). The entire protein has a predicted theoretical isoelectric point (pI) of 4.63. The amylase gene appears to be divided into two main regions, joined at the *Bam*HI site. The first 5′ half, corresponding to the first 480 amino acids, revealed typical features of the well-characterized α-amylase family (Rogers, 1985). The greatest similarity was found with a limited number of α-amylases: (i) 96.8% identity in 155 amino acids overlaps with the partial sequence of α-amylase from *L. amylovorus* (Fitzsimons et al., 1994); (ii) 64.5% identity in 462 amino acids overlaps with the α-amylase of *B. subtilis* (Yang et al., 1983); (iii) 27.2% identity in 430 amino acids overlaps with the α-amylase of *Butyrivibrio fibrisolvens* (Rumbak et al., 1991).

The 3′ half displays an unusual structure consisting of tandem repeated sequences; it consists of four perfectly homologous 104 amino acid repeats, each of them beginning with a microsatellite-like structure (agc agc agc agt agt agt aca aca aca). This microsatellite-like structure has been well documented in eukaryotic genomes (Tautz and Renz, 1984; Bruford and Wayne, 1993), but, to the best of our knowledge, is extremely rare in prokaryotic genomes. Furthermore, a perfectly repeated octanucleotide sequence caagcagc is found at the borders of each repeat, suggesting an insertion event followed by duplication in an ancestral α-amylase gene.

This repeated region was used as a probe in Southern hybridization analyses. The same pattern as for the 371-bp probe (data not shown) was observed, indicating

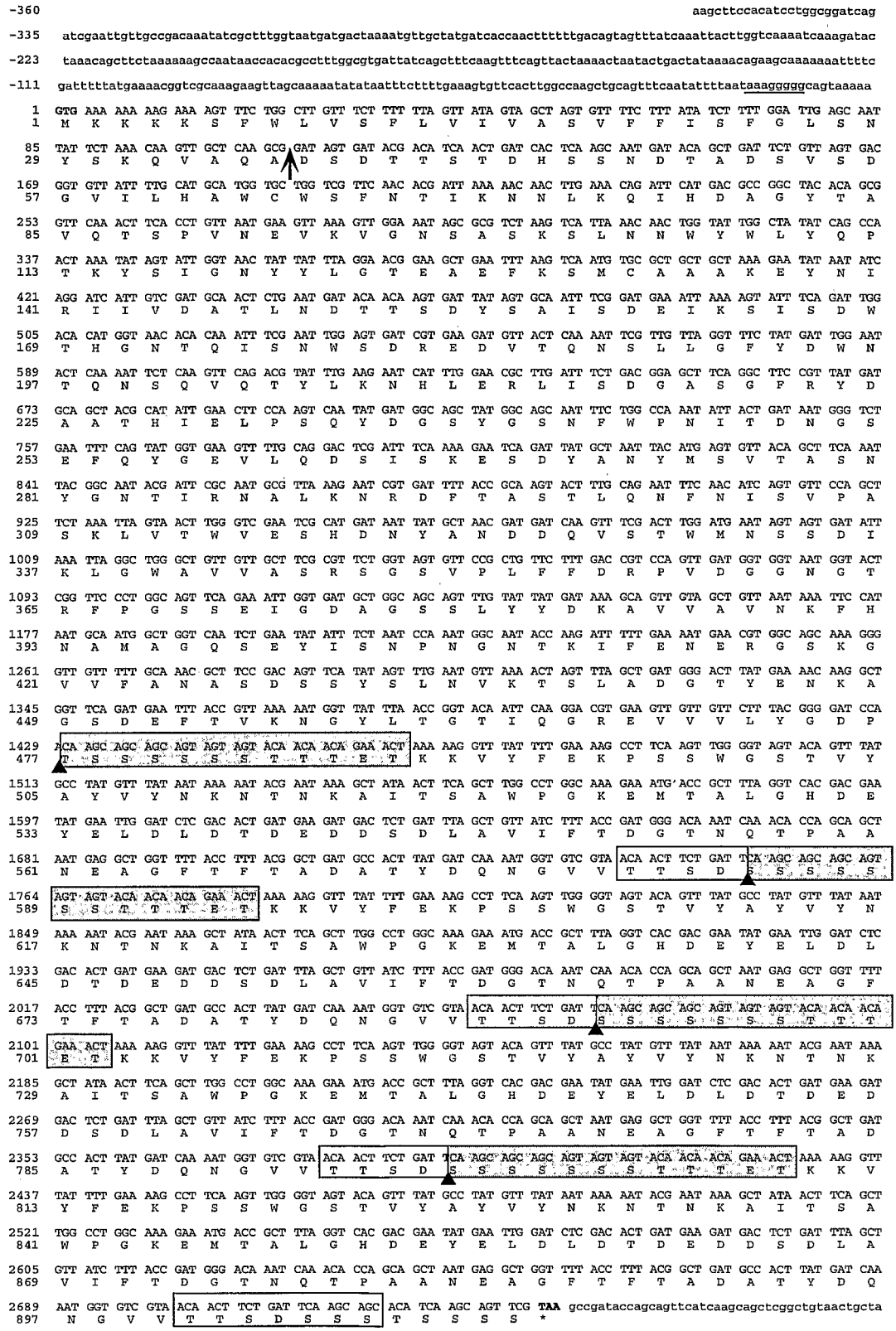

Fig. 2. The nucleotide sequence and the deduced amino acid sequence of the *amy*A of *L. plantarum* A6. Putative ribosome-binding sites (underlined), start and stop codon (boldface) are indicated. The presumed signal peptidase cleavage site is indicated with an arrow. The beginning of each repeated sequence is highlighted with a triangle. The 5′-end flanking regions of the 'consensus' repeat unit are shaded and the 3′-end flanking regions of the 'consensus' repeat unit are boxed.

that these repeats are always associated with α-amylase genes and that other similar repeats are not dispersed in the chromosome.

3.4. *Nucleotide sequence of the* L. amylovorus amyA *gene*

The strong homology between the partially sequenced α-amylase gene of *L. amylovorus* and the corresponding region in *L. plantarum* A6 led us to perform full characterization of the former.

Southern hybridization analyses of the *L. amylovorus* chromosomal DNA were carried out using the same restriction enzymes and the same probes as for *L. plantarum*. A main band was observed for each digest (Fig. 1) suggesting that the *amy*A gene is unique in *L. amylovorus*. It is interesting to note that four out of six digests yield very similar fragment sizes in the two different species. Thus, it was decided to use the primers defined for the sequencing of the *amy*A gene of *L. plantarum* A6 to isolate and sequence the *amy*A gene of *L. amylovorus*.

A 2862 bp long open reading frame (*amy*A) encoding a protein of 954 amino acids with a molecular mass of 104 674 Da was identified (Fig. 3). Perfect homology between the two *amy*A genes can be observed in the first 5′ half (up to the *Bam*HI site) corresponding to the active site, with only seven mismatches in 1600 nucleotides.

As for *L. plantarum* A6 *amy*A, the second part shows the presence of tandem repeat units but striking differences are observed (Fig. 4): (i) Five repeat units are present in *L. amylovorus* as opposed to four in *L. plantarum*. (ii) The repetition unit is shorter (273 nucleotides instead of 321). These repeat units encode 91 amino acids and are homologous with each other and show only five mutations with the corresponding part of the *L. plantarum* A6 repeat unit.

The comparison of repeated sequences of the two *amy*A genes led us to define a 'consensus' repeat unit of 273 nucleotides bordered by two regions: (i) a 5′ end flanking region of 35 nucleotides containing the octanucleotide insertion site and the microsatellite-like structure; (ii) a 3′ end flanking region of 21 nucleotides containing the octanucleotide insertion site. These flanking regions are found at the extremity of each repeat unit of 273 nucleotides for *L. plantarum* A6, but only once at the borders of the tandem repeated region of *L. amylovorus* (Fig. 2, Fig. 3 and Fig. 4). Note the deletion of the two trinucleotides agt in the microsatellite-like structure of *L. amylovorus*.

The particular structure of the *amy*A genes and the comparison of the tandem repeats suggest the insertion of a 321 nucleotides sequence in an ancestral gene of α-amylase, followed by dispersion in the two *Lactobacillus* species with duplications specific to each strain.

4. Discussion

The molecular characterization of the *amy*A genes of *L. plantarum* A6 and *L. amylovorus* has been described. A very high homology between the two genes is observed, raising the question of the correct identification of the A6 strain. We have sequenced a highly variable region corresponding to the first 400 bp of the 16S rRNA of the A6 strain, and have found a perfect similarity with the *L. plantarum* 16S rRNA sequence (Woese et al., 1992, unpublished). This last result confirms without any ambiguity the identification of the A6 strain to the species *L. plantarum*.

The amino acid sequences deduced indicate that both *amy*A genes encode a large polypeptide with a molecular weight of about 100 kDa. These polypeptides are approximately twice as large as typical microbial α-amylases (50–60 kDa) (Vihinen and Mäntsälä, 1989). However, they are smaller than the estimated molecular weights of the corresponding purified proteins. The amylase protein of *L. amylovorus* has been estimated to be 126 kDa (Jore and DeParasis, 1993) or 150 kDa (Iman et al., 1991). SDS-PAGE analysis of the purified α-amylase of *L. plantarum* A6 resulted in a well-defined band (50 kDa) and a diffuse band close to 150 kDa (Giraud et al., 1993). The discrepancies in the estimation of the size can been explained either by migration artefacts of the proteins in SDS-PAGE, as has been reported for some amylases (Robyt and Ackerman, 1973; Arakawa et al., 1992), or by glycosylation of the protein, as observed for bacterial amylases such as those of *Alicyclobacillus acidocaldarius* (Schwermann et al., 1994), or *Bacillus brevis* (Stefanova and Emanuilova, 1992).

A remarkable feature of the structure of both *amy*A genes is the presence, at the 3′ end, of direct tandem repeat sequences which share 100% similarity with each other. A striking difference is that the repetition number and the size of repeats vary between the two organisms: four repeated sequences of 321 nucleotides for the *amy*A of *L. plantarum* A6, five repeated sequences of 273 nucleotides for *L. amylovorus*. Southern hybridization analysis showed another noticeable difference between the strains. Whereas only one copy of the *amy*A gene is observed for *L. amylovorus*, two copies were revealed in *L. plantarum* A6. For all the digestion, except for *Pst*I, the size differences between the two copies were always in the same range (600–700 bp). It can be suggested that both gene copies observed in *L. plantarum* A6 differ by only two 321-bp repeat units and that these duplicated *amy*-genes are located within a single large *Pst*I fragment.

We have investigated by PCR experiments the presence of such repeated sequences in other lactic acid bacteria (*Lactococcus lactis* ATCC 11454, *Leuconostoc mesenteroides* ATCC10832, *Pediococcus pentosaceus*

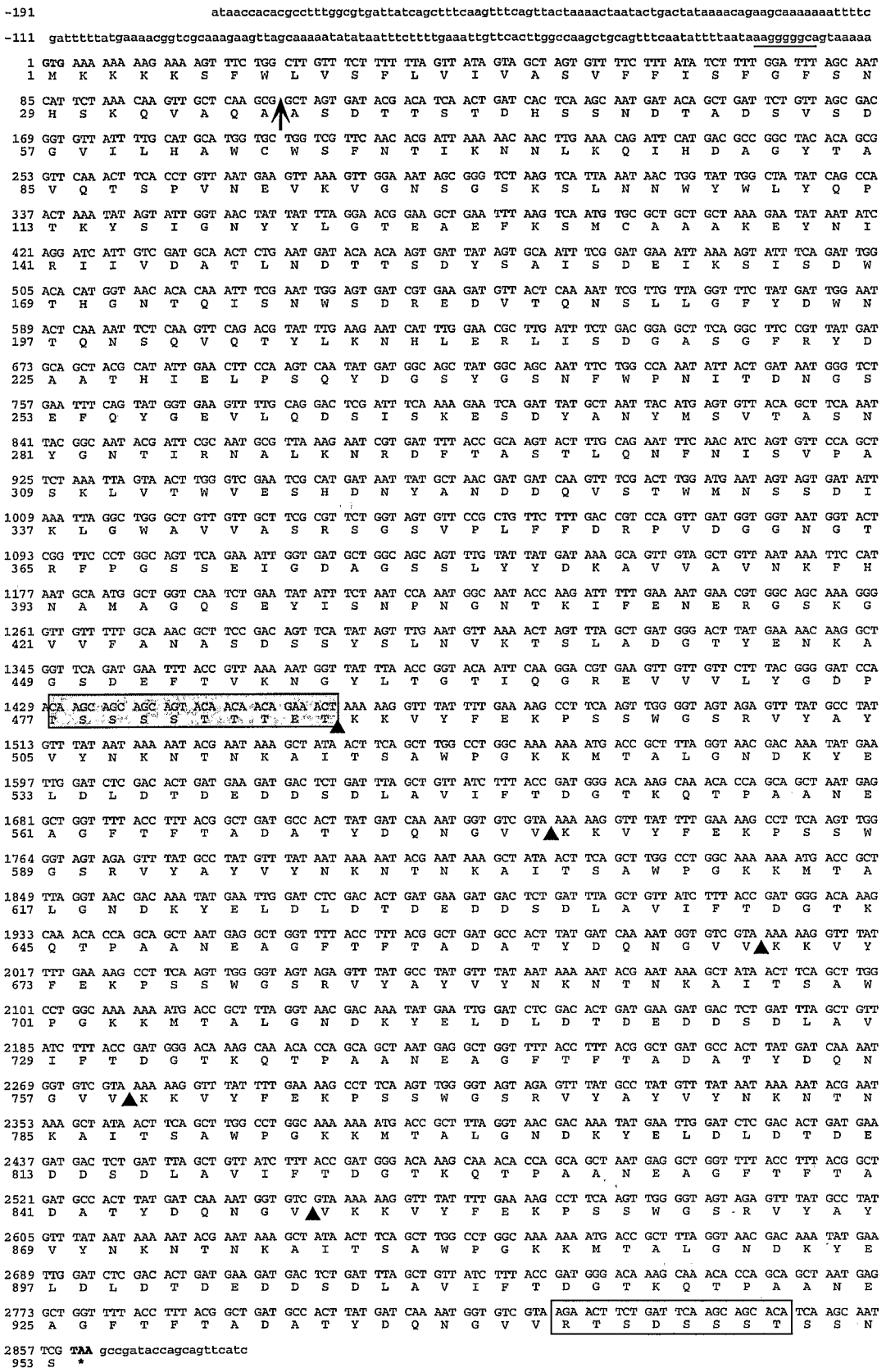

Fig. 3. The nucleotide sequence and the deduced amino acid sequence of the *amy*A of *L. amylovorus*. Putative ribosome-binding sites (underlined), start and stop codon (boldface) are indicated. The presumed signal peptidase cleavage site is indicated by an arrow. The beginning of each repeated sequence is highlighted with a triangle. The 5′-end flanking region of the 'consensus' repeat unit is shaded and the 3′-end flanking region of the 'consensus' repeat unit is boxed.

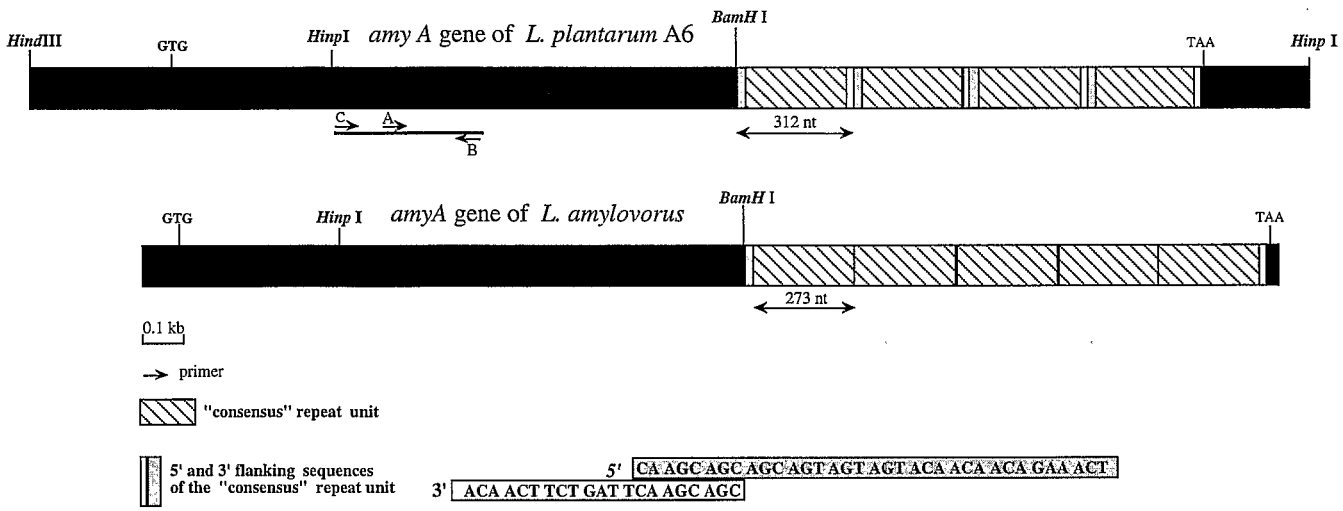

Fig. 4. Diagrammatic sketch of the α-amylase genes of *L. plantarum* A6 and *L. amylovorus*.

ATCC 43200) and in other *L. plantarum* strains (ATCC 14917, ATCC 10241 and DSM 20174). No amplification products were obtained for all the strains tested; these repeat sequences appear specific to *L. plantarum* A6 and *L. amylovorus*.

Jore and DeParasis (1993), using a series of deletion derivatives in the 3′ end of the α-amylase gene of *L. amylovorus*, reported that the N-terminal part (45 kDa) still displays full α-amylase activity. Likewise, Fitzsimons et al. (1994) have demonstrated that the first 590 nucleotides of the *L. amylovorus amy*A gene, including the promoter region, are sufficient to transfer an amylolytic activity to a strain of *L. plantarum* which naturally lacks such activity. The fact that more than 50% of the C-terminal part of the α-amylase is not essential for amylolytic activity raises the question of the function of this region.

The amylases from *B. subtilis*, *L. amylovorus*, and *L. plantarum* A6 have identical physico-chemical properties (same optimal pH and optimal temperature) (Welker and Campbell, 1967; Fogarty, 1983; Giraud et al., 1993; Pompeyo et al., 1993). Nevertheless, only *L. plantarum* A6 and *L. amylovorus* possess the special ability to break down raw starch (Giraud et al., 1994; Iman et al., 1991). It is therefore tempting to suggest that the particular structure of the C-terminal half of the α-amylases of these two strains is associated with effective substrate binding. This hypothesis is supported by the description of tandem repeat unit located in the C-terminal portion of a family of clostridial and streptococcal ligand-binding proteins (Wren, 1991). This group of proteins includes toxins from *Clostridium difficile* (von Eichel-Streiber et al., 1992), glycosyltransferases from *Streptococcus* strains (Giffard and Jacques, 1994) and toxin from *Streptococcus pneumoniae* (Yother and Briles, 1992). This family of ligand-binding proteins appears to display a modular design, with one module providing enzymatic functions and the other module consisting of a repetitive carbohydrate-binding domain located in the C-terminal region (Wren, 1991).

The high degree of homology in the DNA sequences for the two α-amylase genes suggests that these genes are evolutionary related to each other. The structural homologies, particularly in the 3′ end part, indicate that both genes probably have a common ancestor and may have evolved independently by duplication and subsequent recombination and mutation, as has been reported for streptococcal glycosyltransferases (Wren, 1991; Giffard and Jacques, 1994) and for *Clostridium difficile* toxins (von Eichel-Streiber et al., 1992). The significant phylogenetic distance between the two *Lactobacillus* strains (Collins et al., 1991) led us to consider that the acquisition of this unusual amylolytic property in *L. plantarum* A6 results from a lateral transfer of this ancestor common gene. Since the repeated sequences are perfectly homologous in one strain and quite well preserved between the two strains, it can be assumed that such an event occurred relatively recently.

The role of these repeated sequences remains to be elucidated. The expression of the entire gene and/or 3′-end truncated forms may confirm their importance in raw starch binding.

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Molecular characterization of the α-amylase genes of *Lactobacillus plantarum* A6 and *Lactobacillus amylovorus* reveals an unusual 3′ end structure with direct tandem repeats and suggests a common evolutionary origin

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