Insertion Elements on Lactococcal Proteinase Plasmids

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DNA segments of 809 and 808 nucleotides, with 18-base-pair terminal inverted repeats, are present on the proteinase plasmids pWW05 from Lactococcus lactis subsp. cremoris Wg2 and pSK111 from L. lactis subsp. cremoris SK11, respectively. These DNA segments are highly similar: 77% identical nucleotides and both contain an open reading frame that can encode a protein of 226 amino acids. Furthermore, both DNA segments are located downstream of the proteinase maturation gene prtM, but they differ individually in their orientation with respect to the prtM gene. On the basis of the striking similarity between ISS1, an 808-base-pair insertion sequence (IS) from L. lactis subsp. lactis ML3 lactose plasmid pSK08, and the DNA segments of pWW05 and pSK111, we propose that these DNA segments comprise IS elements. The IS elements from strains Wg2 and SK11 were named ISS/J and ISS/I, respectively. On pWW05, ISS/J is flanked on one side by only part of a second IS element, indicating that pWW05 evolved as a deletion derivative of a precursor plasmid that carried at least two IS elements.

In lactococcus (formerly called lactic streptococcus [25]) research, conjugal transfer of plasmids is an important genetic tool. A number of traits of economic importance, such as proteinase production, fermentation of lactose and citrate, and bacteriophage resistance, as well as the production of antagonistic compounds such as bacteriocin and nisin, were demonstrated to be plasmid encoded by conjugation (11). During the last few years, it has become evident that the frequency of conjugation is often dependent on transposition-mediated cointegrate formation between conjugative and nonconjugative plasmids.

As described by Anderson and McKay (1), upon conjugal transfer, the 55-kilobase (kb) lactose plasmid pSK08 from Lactococcus lactis subsp. lactis ML3 forms a 104-kb cointegrate with the 48.4-kb conjugal plasmid pRS01. Since the replicon fusion, which introduced a 0.8- to 1-kb fragment of DNA into the junction fragment, was shown to be independent of general recombination, the presence of an insertion sequence (IS) on the lactose plasmid was proposed. This hypothesis appeared to be correct on the basis of determining both the nucleotide sequences of the junction fragments of pRS01::pSK08 cointegrate and the nucleotide sequences of the corresponding regions of the initial precursor plasmids. In this way, Polzin and Shimizu-Kadota were able to identify two 808-base-pair (bp) iso-IS elements: ISS/J and ISS/T (22). The presence of an IS element on 54-kb proteinase-lactose plasmid pUCL22 from L. lactis subsp. lactis Z270 was demonstrated by Novel et al. (20). Plasmid pUCL22 formed a cointegrate with the 30-kb heterologous conjugative plasmid pVA797. Upon resolution of the cointegrate, the plasmid corresponding to pVA797 was enlarged by 1.2 kb. Similarly, Higgins et al. (14) demonstrated that upon resolution of a pTN20::pTR1040 cointegrate following conjugal transfer, the 71.7-kb lactose plasmid pTR1040 was enlarged by 3.3 kb, suggesting the presence of an IS element on the self-transmissible 28.4-kb restriction and modification plasmid pTN20. Recently, D. van der Lelie (Ph.D. thesis, University of Groningen, Groningen, The Netherlands) demonstrated that the nonconjugative plasmid pGL3 became conjugally transmissible upon insertion of an IS element into this plasmid.

For proteinase activity to occur in lactococci, the presence of both a gene encoding a proteinase, prtP, and a gene encoding a maturation protein, prtM, is essential (13, 32). Both genes are present on the proteinase plasmids of L. lactis subsp. cremoris Wg2 (pWW05) and SK11 (pSK111). On both plasmids, both genes are divergently transcribed from regulatory sequences contained within a 0.3-kb ClaI fragment (15, 31). The nucleotide sequences downstream of the prtM genes were not followed by a terminator-like sequence. Instead, in both strains we found an additional open reading frame (ORF), preceded by a ribosome-binding site and a putative promoter sequence (13, 32). As described in this paper, further elucidation of the nucleotide sequence downstream of prtM revealed the presence of an ISS/J-like IS element on the proteinase plasmids from both strain Wg2 and strain SK11.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture medium. The proteinase-producing (Prt+) strain L. lactis subsp. cremoris SK11 (9), as well as the Prt+ and proteinase-deficient (Prt−) variants of L. lactis subsp. cremoris Wg2 (21), were grown on M17 broth supplemented with 0.5% glucose (29). Escherichia coli JM101 was used as a host for M13mp18, M13mp19, pUC18 (33), and their derivatives. Plasmid pGKV500 contains the major parts of both the structural proteinase gene prtP and the proteinase maturation gene, prtM, from L. lactis subsp. cremoris Wg2 (15, 16).

Molecular cloning and DNA sequence analysis. Plasmid DNA was isolated from the lactococcal strains essentially by the method of Birnboim and Doly (3). General molecular cloning techniques were carried out as described by Maniatis et al. (18). DNA fragments from the 27-kb proteinase plasmid pWW05 from strain Wg2 were cloned in pUC18 (Fig. 1A). DNA fragments from the 71-kb proteinase plasmid pSK111 from strain SK11 were cloned in phages M13mp18 and M13mp19 (Fig. 1B). Nucleotide sequences were determined by sequencing either single-stranded phage M13 DNA

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or double-stranded plasmid DNA in two orientations by the dideoxy-chain-termination method (24), using the T7 Sequencing Kit (Pharmacia, Uppsala, Sweden). Synthetic 17-mer DNA primers were prepared on a DNA synthesizer (model 380A; Applied Biosystems, Foster City, Calif.) and were kindly provided by Unilever Research Laboratories, Vlaardingen, The Netherlands. Restriction enzymes were purchased from Boehringer GmbH, Mannheim, Federal Republic of Germany.

Southern blotting and hybridization. Plasmids and DNA fragments separated by electrophoresis in a 0.8% agarose gel were transferred to GeneScreen Plus filters essentially by the method of Southern (27), as modified by Chomczynski and Qasba (5). DNA probes were labeled with 20 μCi of [α-32P]dCTP (3,000 Ci/mmol; Amersham International, United Kingdom) by using a Random Primed DNA-labeling kit (Boehringer GmbH). Following hybridization and washing as described by Church and Gilbert (6), the filters were exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) for autoradiography.

RESULTS

Nucleotide sequence analysis. A 678-bp ORF, ORF-N1, 149 bp downstream of the prtM gene, was found on proteinase plasmid pSK111 from strain SK11. The TAA stop codon of this ORF was directly followed by stop codons in all three possible reading frames (Fig. 2). ORF-N1 is contained within a 772-bp DNA segment which is flanked by an 18-bp inverted repeat (Fig. 1 and 2).

On proteinase plasmid pWV05 from strain Wg2, also 149 bp downstream of the prtM gene, a 168-bp ORF, ORF-W1, was found (Fig. 1 and 3). The ATG start codons of both ORF-N1 and ORF-W1 are preceded by typical promoterlike −35 and −10 sequences and a putative ribosome-binding site (8). The 149-bp DNA segments separating prtM and ORF-N1 or ORF-W1 and containing the putative expression signals are identical in both strains. Furthermore, the first 113 bp of ORF-W1 and ORF-N1 was identical, except for two nucleotides. However, no further homology between ORF-N1 and the nucleotide sequence directly downstream of ORF-W1 could be found. The first 113 bp of ORF-W1 is flanked by two stretches of 18 bp which form an imperfect direct repeat, with only one mismatch (Fig. 3). The 18-bp repeats present upstream of ORF-W1 and ORF-N1 contain the −35 sequence of the putative promoter. The start of the second 18-bp direct repeat, present only on pWV05 in Wg2, marks the position where homology between ORF-W1 and ORF-N1 ends. This 18-bp repeat within ORF-W1 forms an imperfect inverted repeat with a stretch of 18 nucleotides, 900 bp downstream of the ORF-W1 start codon (Fig. 3). This inverted repeat flanks a 773-bp DNA fragment, containing a 678-bp ORF oppositely oriented with respect to ORF-W1 and prtM. We designate this ORF ORF-W2 (Fig. 1). The ATG start codon of ORF-W2 is, like those of ORF-W1 and ORF-N1, preceded by typical promoterlike −35 and −10 sequences and a putative ribosome-binding site.

Homology comparison. The deduced amino acid sequences of ORF-W2 and ORF-N1 are of equal length, 226 amino acids, and contain 85% identical residues. When considering conservative amino acid substitutions, the overall homology amounts to 91%.

The proteinase plasmid from strain Wg2 contains a DNA segment of 809 nucleotides, the ends of which are formed by 18-bp inverted repeats and which comprise ORF-W2. This 809-bp segment is designated w2. The corresponding segment of DNA from strain SK11, containing 808 bp with 18-bp terminal inverted repeats and ORF-N1, is designated n1.

Although oppositely oriented with respect to the prtM gene, w2 and n1 show extensive sequence similarity: 77% of the nucleotides are identical. The finding that w2 and n1 both contained a 678-bp ORF and 18-bp terminal inverted repeats prompted us to compare w2 and n1 with the nucleotide sequence of the lactococcal IS element ISSI. ISSI is an 808-bp IS element with 18-bp inverted repeats at its ends and carries a 678-bp ORF. It is present in two versions, ISSI/S and ISSI/T, differing in 4 bp, on the lactose-proteinase plasmid pSK08 from L. lactis subsp. lactis ML3 (22). This comparison showed that the ISSI/ nucleotide sequence showed 76 and 84% similarity with the nucleotide sequences of w2 and n1, respectively. Comparison of the protein of 226
proteinase activity (19). When analyzed by sequence similarity with the C-terminal ORF-N1, the analogous sequences from other lactococcal strains, 60% identity was found in addition to the 27-kb proteinase plasmid pWV05 of the 6.5-kb BglII fragment from pWV04.

Similarly, w2 and n1 correspond 51 and 50%, respectively, with the nucleotide sequence of IS26 (19). When comparing w1 and n1 with the nucleotide sequence of Bacillus thuringiensis IS element IS240, a similarity of 57 and 55%, respectively, was found (7). An even higher degree of nucleotide sequence similarity was found when comparing w2 and n1 with the Staphylococcus aureus IS element IS431L, a member of the IS257 family of IS elements: 62 and 60%, respectively (2, 23). The largest ORF present on IS431L can encode a protein of 224 amino acids. This putative protein contains 60 and 58% identical residues when compared with the amino acid sequences deduced from ORF-W2 and ORF-N1, respectively (Fig. 4). If conserved amino acid substitutions are taken into account, both ORF-W2 and ORF-N1 show 71% homology with the largest ORF present on IS431L. The striking sequence similarity between IS51 and the S. aureus IS257 family of IS elements was described previously by Roux and Skurray (23).

Southern hybridization. Proteinase-producing L. lactis subsp. cremoris Wg2 (Prt+) contains, in addition to the 27-kb proteinase plasmid pWV05, the following four other plasmids: pWV04 (18 kb), pWV03 (9.5 kb), pWV02 (4.5 kb), and pWV01 (2.2 kb). In the proteinase-deficient variant of L. lactis subsp. cremoris Wg2 (Prt−), pWV05 and pWV03 are absent (21). In order to determine whether plasmid pWV05 contained additional copies of DNA sequences similar to those of w2, plasmid DNA from both the Prt− and Prt+ variants of strain Wg2 was hybridized with a 1.7-kb BglII-AccI DNA fragment from pWV05, containing segment w2. Plasmids pWV05 and pWV04 both hybridized with this probe (Fig. 5C, lanes 1 and 4). DNA fragments from either plasmid hybridizing with this probe were readily distinguishable when the signals from both strains were compared. On the basis of this comparison, the 15-kb EcoRI fragment (Fig. 5C, lanes 2 and 5) and the 3.3-kb fragment in the BglII-HindIII digest (lanes 3 and 6) must have originated from pWV04, whereas the 7.0-kb EcoRI fragment (lane 5) and the 8.5-kb BglII-HindIII fragment (lane 6) must have originated from pWV05. The sizes of the hybridizing DNA fragments from pWV05 indicate that pWV05 carries no further copies of DNA fragments homologous to either w2 or ORF-W1.

The control experiment shown in Fig. 5B, in which the plasmid content of Wg2 (Prt+) was hybridized to the 6.5-kb HindIII fragment from pGKV500 containing the major part of the L. lactis subsp. cremoris Wg2 proteinase gene, was included to distinguish between pWV05 and pWV04. In addition to the presence of s1- or w2-like sequences on plasmid DNA, there are also copies of these DNA sequences present on the chromosomal DNA of various lactococcal strains. At least two copies of contiguous sequences that hybridize with n1 and w2 are present on the chromosome of the well-studied L. lactis subsp. lactis model strain, MG1363 (results not shown).
FIG. 3. Nucleotide sequence of a 1,200-bp DNA segment from proteinase plasmid pWV05 of strain Wg2. The numbering of the nucleotides starts at the HindIII site. The 18-bp direct and inverted repeats are indicated by dashed arrows. The single mismatch in the 18-bp inverted repeat is marked by a plus. The deduced amino acid sequences of ORF-W1 and ORF-W2 and the C-terminal part of PrtM are shown with the sequence. Asterisks represent stop codons. The putative -35 and -10 sequences, as well as the putative ribosome-binding sites (rbs), are indicated.

DISCUSSION

On the proteinase plasmids from L. lactis subsp. cremoris Wg2 and SK11, we found two homologous DNA segments of 809 and 808 bp, respectively, with 18-bp inverted repeats at their ends. These segments were labeled w2 and n1. The 18-bp inverted repeats in n1 were identical to the 18-bp inverted repeat of IS element ISSI from L. lactis subsp. lactis ML3 lactose-proteinase plasmid pSK08, while the repeats in w2 contained one mismatch (22). A nucleotide sequence homology comparison of w2 and n1 with ISSI revealed a high degree of homology: 76 and 84% for w2 and n1, respectively. An even higher degree of homology was present when the amino acid sequences deduced from the largest ORFs present on w2 and n1 were compared with that from the largest ORF present on ISSI: 86 and 93%, respectively. All ORFs mentioned have the potential to specify a protein of 226 amino acids. On the basis of the striking similarity between w2, n1, and ISSI, we propose that DNA segments w2 and n1 represent IS elements. Since w2 and n1 are extremely homologous to ISSI and, like ISSI, originate from lactococci, the IS elements from L. lactis subsp. cremoris Wg2 and SK11 will be designated ISS/W and ISS/N, respectively.

On proteinase plasmid pWV05 from strain Wg2, in between prtM and ISS/W, a 186-bp DNA fragment which is identical to the first 186 nucleotides of ISS/N, except for two mismatches, is present (Fig. 3). This DNA fragment most probably originated from an additional IS element on the Wg2 proteinase plasmid, identical to ISS/N with respect to localization and orientation relative to prtM. Hybridization experiments showed that apart from ISS/W and the remainder of this second IS element, pWV05 did not carry any other copies of ISSI-like elements.

FIG. 4. Alignment of the deduced amino acid sequences of the largest ORFs present on the following IS elements: ISSI from strain ML3 (22), ISS/N from SK11, ISS/W from Wg2, and ISS31 from S. aureus (2). Only the amino acids differing from the residues in ISSI are shown. Identical residues are boxed.
FIG. 5. Southern hybridization. (A) Plasmid DNA from proteinase-producing (Prt\textsuperscript{+}) (lanes 1 through 3) and proteinase-deficient (Prt\textsuperscript{-}) (lanes 4 through 6) variants of WG2, which were undigested (lanes 1 and 4), digested with EcoRI (lanes 2 and 5), or digested with BglII and HindIII (lanes 3 and 6). (B) DNAs hybridized with a 6.5-kb HindIII fragment from pGKV500 (14), containing major parts of the genes prtP and prtM. (C) DNAs hybridized with a 1.7-kb BglII-Accl fragment from pWV05 containing ISS/W. The positions of the plasmids pWV01, pWV02, pWV03, pWV04, and pWV05 are marked in the margin on the left. Phage SPFI DNA digested with EcoRI was used as a size marker (A, unmarked lane).

The evolution of a plasmid like pWV05, carrying an IS element followed by only part of a second IS element, may be explained in two different ways. In both models we assume the presence of two oppositely directed IS elements on the same replicon, as is the case for lactose-proteinase plasmid pSK08 from _L. lactis_ subsp. _lactis_ ML3 (22). The first model (Fig. 6) envisages the insertion of a third IS element, located either on a plasmid or on a chromosome (Fig. 6, B), into a target DNA sequence within one of the IS elements on plasmid A. This event will result in cointegrate formation (Fig. 6, C) or chromosomal integration of plasmid A. Subsequent homologous recombination between the two copies of the newly inserted element would result in a plasmid with one copy of the donated IS element within a second IS element (Fig. 6, D). Homologous recombination between the two different but strongly related IS elements, in direct orientation, on either plasmid C or plasmid D will result in the formation of a deletion derivative of plasmid A. Plasmid A thus carries a hybrid IS element followed by only part of the second IS element (Fig. 6, E). When chromosomal localization of the donor IS element (Fig. 6, B) is assumed, this homologous recombination will leave part of plasmid A on the chromosome. Alternatively, according to the model for replicative transposition as proposed by Shapiro (26), one may envisage transposition of an IS element into a target DNA sequence within a second IS element on the same replicon, plasmid, or chromosome. Depending on the orientation of the ligation step, this will result in the formation of two circles, both of which carry a copy of the donor IS element and only part of the target IS element. The overall effect of this intramolecular transposition event will be the deletion of part of the original replicon. According to either model, the presence of one copy of an IS element on proteinase plasmid pWV05 and only part of a second IS element implies that pWV05 is a deletion derivative of a larger precursor plasmid that carried at least two IS elements. Also according to either model, the formation of pWV05 may have been accompanied by the formation of a nonreplicative plasmid carrying at least one IS element. Chromosomal integration by transposition of such a nonreplicative plasmid is conceivable.

As proposed by Steele et al., intramolecular transposition of a transposable element on pJS96 from _L. lactis_ KP3 upon conjugal transfer resulted in the formation of pJS96-derived deletion plasmids (28). The genetic element pJS96 carries the lactose-metabolizing genes and is capable of replication both as an integrated part of the lactococcal chromosome and as a plasmid (28). Intramolecular transposition of an IS element had been previously invoked by Anderson and McKay (1) to explain the formation of deletion derivatives from the _L. lactis_ 712 lactose-proteinase plasmid pLP712. Insertion of an IS element into a target sequence within a second IS element was described before by Trieu-Cuot and Courvalin (30). The gram-negative element IS15 (1,648 bp) appears to be the result of insertion, in direct orientation, of the 820-bp IS element IS15α into itself. The same authors have suggested that the structurally closely related IS26 might have resulted from homologous recombination between two variants of IS15α.

As described in this paper, significant sequence similarity was observed among ISS/W, ISS/N, and ISSI from _L. lactis_ subsp. _lactis_ ML3 (22), IS26 from _P. vulgaris_ (19), IS431 from _S. aureus_ (2), and IS240 from _B. thuringiensis_ (7). This homology is illustrated by the alignment of the amino acid sequences of the putative proteins that may be encoded by
the largest ORFs carried by ISS/W, ISS/J, ISS/J, and IS431 (Fig. 4). On the basis of the high degree of sequence similarity between members of the IS element families—

IS257 (including IS431) from \( S. aureus \), ISS/J from \( L. lactis \), and IS45 (including IS26)—from gram-negative bacteria, Rouch and Skurray (23) have proposed that these IS elements form a superfamily of IS elements, sharing a common ancestor. In this perspective, it might be significant to note that the key enzyme in lactose metabolism in \( S. aureus \) is, as in mesophilic lactococci, phospho-\( \beta \)-galactosidase (4). A. \( S. aureus \) phospho-\( \beta \)-galactosidase was shown to be highly homologous to the plasmid-encoded phospho-\( \beta \)-galactosidase of \( L. lactis \); 82% identical (4, 10). The conserved nature of both the phospho-\( \beta \)-galactosidase genes and the IS elements from \( S. aureus \) and \( L. lactis \) suggests an IS element-mediated conjugal transfer of genes involved in lactose metabolism between these unrelated organisms.

In all lactococcal strains examined so far, the proteinase genes are plasmid encoded (16). In a number of \( L. lactis \) subsp. \( lactis \) and \( L. lactis \) subsp. \( cremoris \) strains, both the lactose and proteinase genes are located on a large (45- to 100-kb) plasmid, whereas in a number of \( L. lactis \) subsp. \( cremoris \) strains, the lactose genes are chromosomally encoded (12). In those lactococcal strains in which no physical linkage exists between these two sets of genes, the proteinase genes are usually encoded by much smaller plasmids (14 to 34.5 kb) (17). As proposed in this paper, the 27-kb proteinase plasmid pWV05 (Pt + Lac) from strain Wg2 may have originated from a larger plasmid carrying at least two IS elements by a transposition-mediated deletion event. It is conceivable that lactococcal strains in which the proteinase genes are plasmid located and the lactose genes are contained in the chromosome have originated from strains in which the two sets of genes were originally located on one plasmid. The physical linkage between these genes could have been disrupted, as pointed out above, in such a way that the lac genes became integrated in the chromosome.

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ADDITION TO THE PROOF

Analysis of 0.5 kb of the nucleotide sequence immediately downstream of the proteinase gene on pSK111 revealed the presence of an IS element in direct orientation with respect to ISS/J. Immediately downstream of the proteinase gene on pWV05, the remainder of an IS element was found on a 112-bp DNA segment, which was 63 and 97% identical to a terminal part of ISS/W and ISS/J, respectively. These new findings stress the ubiquitous nature of IS elements or their remnants on lactococcal plasmids and do not alter the conclusions of the present paper.

LITERATURE CITED

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