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Phage Abortive Infection Mechanism from *Lactococcus lactis* subsp. *lactis*, Expression of Which Is Mediated by an Iso-IS*S1* Element

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A 5-kb DNA fragment conferring a phage abortive infection phenotype (Abi$^+$) has been cloned from *Lactococcus lactis* subsp. *lactis* IL416. The Abi$^+$ determinant was subcloned on a 2-kb fragment which carried an Iso-IS*S1* element and an open reading frame of 753 bp designated ORFX. Deletion within ORFX entailed the loss of the Abi$^+$ phenotype, establishing that ORFX is the structural *abi-416* gene. The expression of *abi-416* was shown to be mediated by the Iso-IS*S1* element, which contains a sequence fitting the consensus sequence for gram-positive promoters.

The need to improve phage resistance in lactococcal strains used for cheesemaking has long been recognized. Recent molecular and genetic studies of these microorganisms have given insight into phage defense mechanisms and opened new possibilities for the improvement of lactococcal phage resistance.

Naturally occurring lactococci with a high degree of phage resistance have been shown to possess several types of resistance mechanisms including adsorption interference, restriction and modification, and abortive infection (4, 15, 26). It has been suggested that cheesemaking strains might be improved by introducing several defense mechanisms, acting on a broad range of phages at different steps of their life cycle, into these strains. Such an approach relies on the availability of a number of highly efficient, well-characterized phage resistance mechanisms.

We describe here the determinant of the phage abortive infection phenotype (Abi$^+$) of the highly phage-resistant *Lactococcus lactis* subsp. *lactis* IL416 from our collection. An open reading frame of 753 bp was identified as the structural *abi-416* gene. Its expression is mediated by an Iso-IS*S1* element through an active outward transcription promoter.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, and media. *L. lactis* subsp. *lactis* IL1403 (3) and derivatives were grown in M17 medium (31) in which lactose had been replaced by glucose at 30°C (M17glc). *Escherichia coli* TG1 (11) was grown in Luria-Bertani medium (17) at 37°C. When needed, erythromycin (5 μg/ml for *L. lactis*), azaerythromycin (100 μg/ml for *E. coli*), or ampicillin (50 μg/ml for *E. coli*) was added to the culture medium. Azaerythromycin was kindly made available from PLIVA (Zagreb, Yugoslavia). Phage bIL66 is from our collection and was enumerated as described by Terzaghi and Sandine (31). Phage adsorption and one-step growth experiments were performed as previously described (10).

Molecular cloning. Plasmid and total DNAs from *L. lactis* were prepared as previously described (28). In a shotgun cloning experiment, linearized vector molecules and total DNA restriction fragments were mixed at a ratio of 1:3 at a concentration of 100 μg/ml and ligated with T4 DNA ligase (Boehringer Mannheim). Lactococcal protoplasts were transformed with the ligation mixture as previously described (29). The Em^r transformants were subsequently recovered as follows: the colonies embedded in agar medium were dispersed in saline solution by blending with an Ultra-Turax T18/10 (20,000 rpm), and 2 ml of the suspension (ca. 10^6 CFU) was used to inoculate 100 ml of M17glc broth containing 5 μg of erythromycin per ml. After overnight incubation at 30°C, the Em^r, phage-resistant clones were selected as described below. Transformation of *L. lactis* with different plasmid constructs was done by electroporation as described by Holo and Nes (14).

Procedures for DNA manipulation, transformation of *E. coli* cells, and cloning were essentially as described by Maniatis et al. (17).

Selection of phage-resistant transformants. The pool of transformants was serially diluted, and 100 μl of these dilutions was mixed with 50 μl of 1 M calcium chloride and 10^7 phage particles. After 5 min at room temperature, 3 ml of molten M17glc agar containing erythromycin was added to each mixture and poured on the surface of a solidified agar plate with M17glc and erythromycin. Control plates were made with a culture of strain IL1403 without erythromycin.

Southern hybridization. Southern hybridizations were as described by Maniatis et al. (17). DNA probes were prepared with a nick translation kit (Amersham Corp., Arlington Heights, Ill.) and [α-^{32}P]dCTP (Amersham).

DNA sequence analysis. *E. coli* clones for sequencing were obtained by subcloning specific DNA fragments in the pBluescript plasmids (Stratagene, La Jolla, Calif.) and by using DNase I to generate a series of overlapping clones. Single-stranded DNA was prepared as described by Vieira and Messing (32) with MK07 helper phage and was sequenced essentially in accordance with the protocol accompanying the 370A DNA sequencer (Applied Biosystems, San Jose, Calif.). In brief, the DNA was used in dideoxynucleotide chain termination sequencing reactions with *Taq*

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DNA polymerase and fluorescent dye-coupled primers (Promega). The reported sequence was determined on both strands. The DNA and protein sequences were analyzed with the BISANCE and the University of Wisconsin Genetics Computer Group software packages, implemented at the Centre InterUniversitaire d'Informatique à Orientation Biomédicale, Paris, France.

Nucleotide sequence accession number. The GenBank, EMBL, and DDBJ nucleotide sequence accession number is M77708.

RESULTS

Cloning a determinant for phage resistance from *L. lactis* subsp. *lactis* IL416. Total DNA from *L. lactis* subsp. *lactis* IL416 was partially digested with *Sau*3AI endonuclease, and fragments of ca. 10 kb were ligated with *Bam*HI-cleaved pIL253 plasmid vector (27) DNA. The ligation mixture was used to transform *L. lactis* subsp. *lactis* IL1403. Twenty randomly isolated Em^r, phage bIL66-resistant transformants were screened for plasmid content, and the phage resistance of the clone carrying the smallest DNA insert was determined by the double-agar-layer method. The recombinant plasmid in this clone carried a 5-kb DNA insert. Further deletion experiments using various restriction endonucleases demonstrated that the phage resistance determinant was present on a ca. 2-kb fragment. Plasmid pIL253 carrying this 2-kb insert was designated pIL611.

Cloned determinant codes for a phage abortive infection mechanism. Phage bIL66 plated on IL1403 forms clear plaques with a mean diameter of 3 mm after overnight incubation. By contrast, when plated on IL1403(pIL611), it forms minute plaques which were only hardly visible after 4 days of incubation. Ninety-seven percent of phage bIL66 particles were adsorbed on IL1403 after 15 min of contact at 30°C. The same efficiency of adsorption was observed with IL1403(pIL611) (data not shown). One-step growth curves obtained with IL1403 and IL1403(pIL611) (Fig. 1) revealed different phage multiplication cycle parameters. The number of infective centers obtained with IL1403(pIL611) was only 0.04% of the input adsorbed phages. The durations of the latent period were similar for the two strains, but the burst size of 150 in IL1403 was reduced to 30 for IL1403(pIL611). In addition, in this later strain, the burst is much more synchronized than in IL1403.

Phages picked up from plaques on IL1403(pIL611) were able to grow on IL1403 but not on IL1403(pIL611), indicating that host-controlled modification was not involved. The phage resistance mechanism encoded by pIL611 is phenotypically identical to previously described phage abortive infection mechanisms (10, 16, 20). This phenotype was designated Abi^+.

DNA sequence analysis of *abi-416*. The nucleotide sequence of the 2-kb DNA insert in pIL611 was determined. An analysis of the sequence shown in Fig. 2 revealed an Iso-IS*S1* element upstream of an open reading frame designated ORFX (250 codons) (Fig. 3). The Iso-IS*S1* element shares 99.5% homology with IS*S1* at the nucleotide level (22), and the protein specified by the transposase gene shares 59.4% homology with IS*257* and IS*431* from *Staphylococcus aureus* (2, 25), 43.6% homology with IS*26* from *Proteus vulgaris* (19), and 43.5% homology with IS*240* from *Bacillus thuringiensis* (5).

We established that ORFX is responsible for the Abi^+ phenotype by deletion of the *Afl*III fragment from position 1043 to 1573 (Fig. 3). When the deleted construct pIL632 was introduced into IL1403, it did not confer the Abi^+ phenotype to the strain. By contrast, a control construct carrying an intact ORFX was Abi^+.

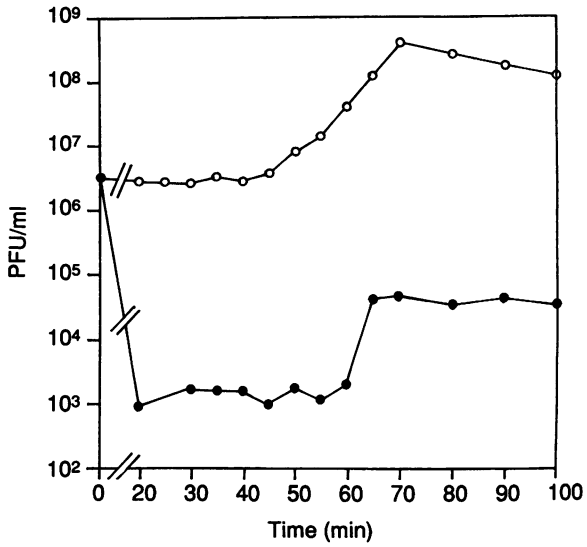

FIG. 1. One-step growth curves of phage bIL66 on *L. lactis* subsp. *lactis* IL1403 (○) and IL1403 carrying pIL611 coding for the Abi^+ phenotype (●). Time zero refers to the beginning of phage adsorption, which was allowed for 15 min at 30°C and then halted by a 10-fold dilution in an antiphage serum. From 20 to 100 min, infective phages were titrated on strain IL1403.

Transcription of the *abi-416* gene is mediated by a promoter within the Iso-IS*S1* element. Examination of the sequence indicated that the only lactococcal promoter-like −35 and −10 sequences (6) upstream of *abi-416* were in the Iso-IS*S1* element (Fig. 2). To test the hypothesis that transcription of *abi-416* is mediated by this region of the Iso-IS*S1* element, two deleted clones constructed during nucleotide sequence analysis which either possessed or lacked this region were compared (Fig. 3). They were first made replicative in *L. lactis* by insertion of plasmid pIL253 and then examined for their ability to confer the Abi^+ phenotype on strain IL1403. The clone carrying pIL627 was Abi^+, whereas that carrying pIL628 was Abi^-. This indicates that transcription of *abi-416* is mediated by a promoter within Iso-IS*S1* (Fig. 3).

It has been recently reported that a chromosomal IS sequence was transposed to a cloned DNA fragment after transformation of *L. lactis* subsp. *lactis* (18, 21). The possibility, therefore, existed that the structure we have described had been generated during the cloning experiment. The DNA hybridization experiment described in Fig. 4 demonstrated that Iso-IS*S1* was present upstream of *abi-416* in strain IL416 and excluded the possibility that the Iso-IS*S1* element was transposed during cloning experiments.

DISCUSSION

A 5-kb DNA fragment from *L. lactis* subsp. *lactis* IL416 coding for a phage resistance phenotype has been cloned. Subcloning experiments allowed us to localize the determinant on a 2-kb fragment. One-step growth experiments established that the resistance mechanism resembles abortive infection, and the phenotype was designated Abi^+. Nucleotide sequence analysis of the subcloned fragment revealed an Iso-IS*S1* element and an open reading frame

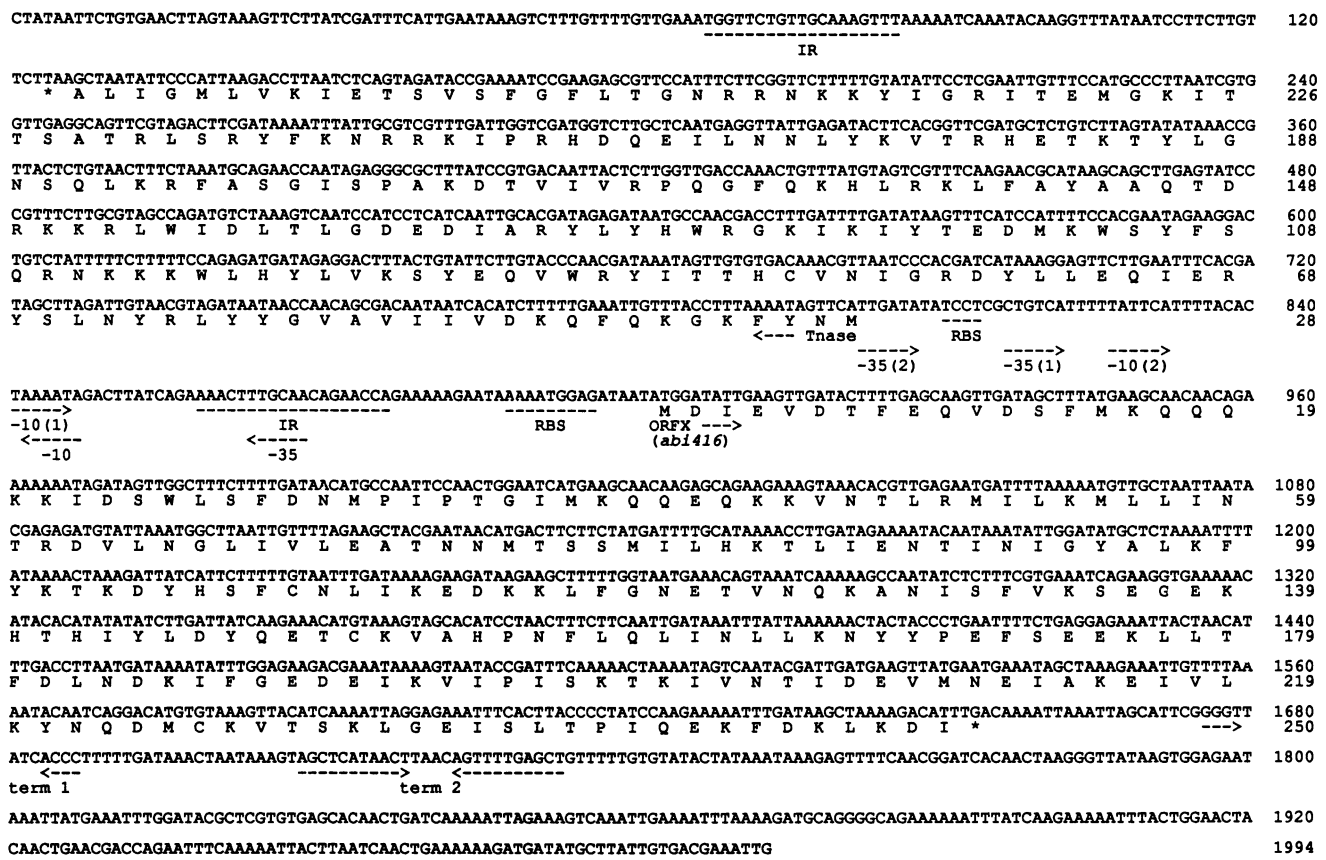

FIG. 2. DNA sequence of the Abi$^+$ phenotype determinant. The deduced amino acid sequences from Iso-IS*S1* transposase (Tnase) and ORFX are indicated. Numbers to the right refer to nucleotides (upper lines) and amino acid residues (lower lines); inverted repeats (IR), potential ribosome-binding sites (RBS), and transcription terminators (term) are indicated by broken underlining of the relevant sequences; potential promoter −35 and −10 boxes are underlined by arrows oriented in the direction of transcription; stop codons are indicated by an asterisk.

designated ORFX. Insertion sequence IS*S1* was first characterized on a lactococcal plasmid coding for lactose utilization (1, 22). Iso-IS*S1* elements were then found downstream of the *prtM* gene on lactococcal proteinase plasmids (12) and on the pTR2030 plasmid which codes for a restriction and modification mechanism and a phage abortive infection mechanism (24). Copies of IS*S1* in the chromosomes of a variety of lactococcal strains (9) have also been identified.

IS*S1* and all the Iso-IS*S1* elements sequenced up to now (12, 22, 24) are 808 bp long except one (809 bp) (12), possess one gene coding for a transposase of 226 amino acids, and are flanked by identical inverted repeats of 18 bp. The Iso-IS*S1* element we described is similar except that the inverted repeats are 1 bp longer (19 bp). These Iso-IS*S1* elements share 79 to 99.5% homology with IS*S1* at the nucleotide level and have a high degree of sequence similarity with IS*240* from *B. thuringiensis*, IS*257* and IS*431* from *S. aureus*, and IS*26* from *P. vulgaris*, which were proposed to form a superfamily of IS elements by Rouch and Skurray (25). Transposition of IS*S1* usually generates 8-bp, directly repeated duplications of the target site flanking the newly inserted element both in the lactose (22) and the pTR2030 (24) plasmids. Only 4-bp direct repeats (GAAA) are found at either end of the Iso-IS*S1* element described in this paper. Variations in target duplication length have been reported for IS*1*, IS*4*, IS*21*, and IS*186* (8) and could also occur for IS*S1*. Another possible explanation could be that the 4-bp repeats are due to chance and that deletion of flanking DNA removed the original direct repeats or that the IS element is associated with a second one to form a composite transposon.

The loss of the Abi$^+$ phenotype following a deletion in ORFX established that ORFX is the structural *abi-416* gene. The protein specified by *abi-416* did not show significant homology to any protein from the data banks, thus preventing any hypothesis being made on its mode of action. In particular, no homology was observed with the protein specified by the *hsp* gene from pTR2030 (13), which specifies a similar Abi$^+$ phenotype, confirming that, as suggested by previous studies on DNA homology (13, 30), different lactococcal genes can encode the phage abortive infection phenotype.

Expression of *abi-416* is mediated by a promoter sequence within the Iso-IS*S1* element. Several insertion elements have been shown to direct expression of downstream genes by the presence of an active outward promoter (8). We have established that this property is shared by IS*S1*. Multiple copies of IS elements within the genome of lactic acid bacteria have been shown to contribute to DNA rearrangements (9). The presence of IS elements close to the lactose (22), proteinase (12), nisin (7, 23), or phage resistance (24)

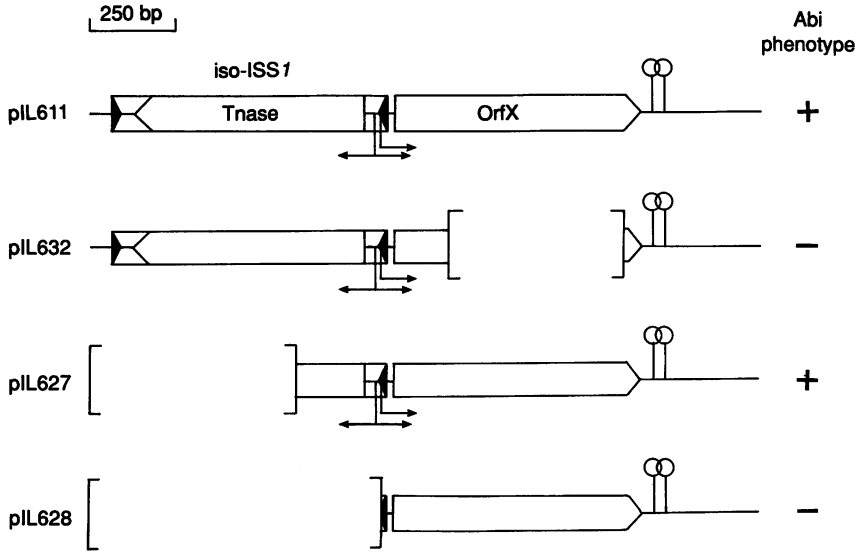

FIG. 3. Structure of the Abi$^+$ phenotype determinant and schematic illustration of its functional characterization. Only the cloned DNA is represented. Iso-IS*S1* is shown as a box, and its inverted repeats are shown as black triangles. The putative promoter and transcription terminator are symbolized as ⊢▸ and ⚲, respectively; deletions are indicated by brackets. Deletion of a 531-bp *Afl*III fragment in ORFX (pIL632) and deletion of a 855-bp fragment (pIL628) which eliminates the putative promoters abolish the Abi$^+$ phenotype. In contrast, deletion of a 603-bp fragment (pIL627) does not abolish the Abi$^+$ phenotype. Deletions were obtained in pBluescript plasmids and plasmid pIL253, subsequently inserted to make the constructs replicative in *L. lactis*.

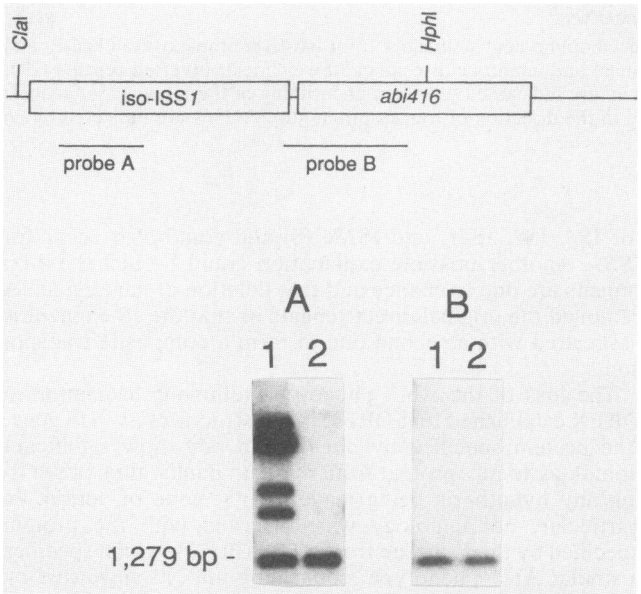

FIG. 4. Comparison of the structure of the Iso-IS*S1* and *abi-416* region in the cloned fragment and in the IL416 strain. A diagram of the cloned DNA fragment is shown at the top. Probes A and B range from nucleotides 168 to 432 and 863 to 1258, respectively (Fig. 2). Total DNA from IL416 (lanes 1) and from the cloned fragment (lanes 2) were cleaved with *Cla*I and *Hph*I endonucleases, electrophoresed, and hybridized with either probe A (A) or B (B). The 1,279-bp *Cla*I-*Hph*I fragment, hybridizing with either probe A or B, was present in the cloned fragment and in the total DNA from IL416, demonstrating that the Iso-IS*S1* and *abi-416* gene region from both origins have the same structure. Additional bands hybridizing with probe B were present in IL416 DNA, indicating that several copies of the IS element are present in this strain.

genes is thought to contribute to the intra- or intercellular mobility of these genes. Our results show that, in addition, these IS elements may contribute to gene expression.

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