Nucleotide Sequence and Distribution of the pTR2030 Resistance Determinant (hsp) Which Aborts Bacteriophage Infection in Lactococci†

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The lactococcal plasmid pTR2030 encodes resistance to bacteriophage attack via two mechanisms, an abortive-infection mechanism, designated Hsp, and a restriction and modification system. We present the complete sequence of the hsp structural gene. The gene is 1,887 base pairs in length and encodes a protein with a predicted molecular mass of 73.8 kilodaltons. The upstream region was cloned in a promoter-screening vector and shown to direct the constitutive expression of the cat-86 gene. An internal probe was used to determine the distribution of the hsp sequence in industrially significant lactococcal strains and to evaluate its relatedness to another lactococcal plasmid implicated in an abortive-infection-type mechanism, pNP40. No homology was detected, suggesting that this gene is not widely distributed in lactococci. Therefore, there are at least two independent abortive-infection genotypes in lactococci.

A number of bacterial plasmids inhibit bacteriophage proliferation by causing cell death prior to the release of viable progeny, a process termed abortive infection (for reviews, see references 1 and 5). The first description of this type of resistance mechanism in lactococci linked it to the conjugal plasmid pTR2030, which completely inhibits plaque formation by small isometric phage (6). A prolate phage (φc2) can form small plaques at a slightly reduced efficiency of plaquing on strains containing pTR2030. A number of plasmids in lactococci which direct similar though not identical phenotypes have been described previously (9).

It has not been determined whether the different phenotypes encoded by these plasmids are the result of different genes or of various degrees of expression of the same basic structural gene. A number of lactococcal plasmids have been shown, like pTR2030, to encode restriction and modification (R/M) activity in addition to abortive infection (2, 9, 11). While this may be taken as presumptive evidence that the plasmids have similar gross genetic organizations, similarity at the gene level or common progenitors should not be presumed. Homology studies have been used to presume relationships between the determinants encoded by both pTR2030 and pC1750, but in this case large fragments were used as probes (11). We show in this study that a small internal gene probe is essential to definitively predict homologies between the different plasmids.

We have undertaken detailed studies of the determinants responsible for both Hsp and R/M. Both R/M and Hsp determinants have been cloned and localized within a 13.6-kilobase (kb) fragment of pTR2030 (3, 4). The hsp locus was defined by Tn5 mutagenesis as a region having a maximum of approximately 3 kb and a minimum of approximately 2 kb (4). This region was shown by deletion analysis to contain all the information necessary to direct the Hsp + phenotype. In this report, we present the complete nucleotide and deduced amino acid sequences of the hsp gene.

Fragments were cloned directionally in M13mp18, M13mp19, and pBluescript to allow sequencing in both directions. The largest pBluescript subclone, with a 2-kb insert, was subjected to ExoIII-mung bean treatment as described by the supplier (Stratagene, La Jolla, Calif.) to generate a set of nested deletions. Nucleotide sequences of both strands were determined by the dideoxy-chain termination method (10) and Sequenase (Stratagene), with either the recombinant M13 single-stranded templates or the pBluescript clones. Synthetic oligonucleotide primers (17-mer) were constructed when a subclone was too large to be fully sequenced from commercially available primers. The facilities of the University of Wisconsin Genetics Computer Group were used to analyze the sequence information.

A physical map of the hsp fragment and the sequencing strategy employed is presented in Fig. 1A. Over 3,000 base pairs (bp) of sequence was determined from the point of Tn5 insertion in plasmid pTRK46, which is the closest insertion that does not inactivate Hsp + (4). This strategy ensures that the total sequence responsible for Hsp has been determined. The sequence was deduced from both strands and multiple runs (Fig. 2). A major open reading frame (ORF) (1,887 bp) from the ATG initiation codon (positions 1105 to 1107) to the ochre termination codon TAA (positions 2989 to 2991) was detected. This is the only possible ORF capable of spanning the four Tn5 insertion sites which inactivate Hsp + (4). The deduced amino acid sequence for this ORF is also presented in Fig. 2. The resulting protein would encode 628 amino acids and have a predicted molecular mass of 73.8 kilodaltons. This is in contrast to previous data which suggested that a 40-kilodalton product is involved in the Hsp + phenotype (4). It is possible that posttranslational processing is responsible for this discrepancy. The upstream region also encodes the carboxy terminus of a large ORF (ORF-X). The Tn5 insertion in pTRK46 disrupted ORF-X without affecting Hsp activity (4). From the data presented above, we conclude that the Hsp + phenotype is directed by a single gene, designated hsp.

It can be predicted that the 1,104 bp of sequence upstream to hsp should contain positive expression signals associated
with the major ORF. This is supported by the fact that the presence of Tn5 in pTRK46 did not alter expression of the Hsp" phenotype (4). Those sequences close to the structural gene that weakly conform to the canonical (three of six at −35 [TTGttt] and five of six at −10 [TAAaAT]) consensus promoter sequences are underlined in Fig. 2. The expression vector pGKV210 (12) was used to analyze the strength of the hsp transcription signals. A clone was constructed in which a 1.4-kb EcoRI-PvuII fragment from pTRK46 containing the upstream sequences and the first 116 bp of the structural hsp gene was inserted in the correct orientation adjacent to the promoterless cat86 gene. This construct, pTRK109, was introduced to Lactococcus lactis NCK203 by electroporation. A representative transformant, NCK271, grew normally in the presence of 12 μg of chloramphenicol per ml. A control strain containing pGKV210 was unable to grow at chloramphenicol concentrations above 1 μg/ml.

Predictions for the secondary structure of the hsp-encoded protein were made with the software program designed by Kyte and Doolittle (7) and included in the University of Wisconsin Genetics Computer Group package. The protein is hydrophilic, with one region of marked hydrophobicity encompassing over 20 amino acids near the amino terminus (positions 80 to 100). This structure presumes a cytosolic globular soluble protein. Searches of the databases (GenBank [version 60; June 1989] and NBRF [version 21; June 1989]) with the algorithm of Wilbur and Lipman (13) did not reveal any significant relatedness between hsp and genes that had been previously sequenced. From the sequence data, it is impossible to predict a function for the hsp-encoded protein based on similarities to existing genes. However, we regard the sequence as the starting point for a greater understanding of the mechanism of action of Hsp. It is also to be expected that this sequence information will enable rapid identification of similar and perhaps, more importantly, different genotypes among the other resistance phenotypes described for lactococci.

Hybridization experiments were conducted to assess whether the abortive-infection mechanism encoded by pTR2030 is genetically related to determinants in other lactococcal strains. Initially a 2.4-kb Sau3A fragment which contains 1,361 bp of the carboxy terminus of the hsp structural gene and approximately 1,000 bp of downstream sequence was used to probe the strain from which pTR2030 originated, *L. lactis* ME2 (6). Multiple homologies were detected in both plasmid and chromosomal sequences (Fig. 3A). However, a probe consisting of an internal 456-bp *XbaI* fragment (positions 2067 to 2523, Fig. 2) cloned in pBlueScript (pTRK73) was specific for the hsp sequence (Fig. 3B). No homology to the control strain N1, which is an ME2 derivative cured of pTR2030, was detected (unpublished data). pNP40 was also digested with *Sau3A* and probed with pTRK73. A strong signal was detected with the pTR2030 positive control, but no homology was observed between the pNP40 genome and the hsp-specific probe (data not shown). This confirms that at least two independent genotypes encode abortive infection in lactococci (11).

Strains (15 *Lactococcus cremoris* and 2 *L. lactis*) were chosen from a bank of strains used either currently or previously by the cheese industry. Of these isolates, 11 were designated phage insensitive and 6 were designated phage sensitive on the basis of screening against commercial phage banks, field performance, and longevity in commercial manufacturing (M. E. Sanders, personal communication). The total DNA content of each of the 17 strains was isolated, digested with *Sau3A*, separated on agarose gels, and probed with pTRK73. No homology was observed between any strain other than ME2, which was included as a positive control. Hsp-like sequences are, therefore, not widely distributed among commercial lactococci. This may be one reason for the effectiveness and longevity of pTR2030 transconjugants once they were reintroduced to the cheese industry (9). The abortive-infection mechanism (Hsp) encoded by this plasmid is one which is not commonly encountered by phages prevalent in this environment.

The importance of using a gene-specific probe in this type of study was emphasized by the results obtained with a probe containing DNA flanking the essential region. Multiple homologies which were not connected to the presence or absence of the hsp gene were detected for pCI750, pTR2030, or pTR2030. The nature of the flanking DNA responsible for the multiple homologies is unclear at this time. It is relevant in this regard that homology was detected between a 13.6-kb pTR2030 fragment containing the hsp gene and the phage resistance plasmid pAJ1106, whereas no homology was evident when the specific hsp probe pTRK73 was employed (A. Jarvis, personal communication). Steele et al. (11) presented hybridization data which suggested a relationship between the resistance determinants encoded by pCI750 (Rbs") and pTR2030. However, we believe that this is not the case, given the extremely weak hybridization signals presented. Homology was also detected between the pCI750 13-kb probe used in the study and a 27-kb EcoRI fragment of pTR2030. This 27-kb EcoRI fragment does not contain the hsp gene (3, 4). It is likely, then, that the rbs determinant of pCI750 is either one of the pNP40 genotypes or a third resistance type.

It is to be expected that detailed analysis of the different abortive-infection determinants will lead to a better understanding of their relatedness, structures, and mechanisms of action. The relationships between different systems will also

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**FIG. 1.** Physical map and sequencing strategy for the 3.0-kb region from pTRK46 encoding Hsp activity. (A) Physical: June showing some of the restriction sites used to generate subclones for sequence analysis. E. *EcoRI*; X. *XbaI*; P. *PvuII*; N. *NcoI*. Symbols: →, direction and length of sequencing runs used to determine the entire sequence in both directions; ■, runs initiated with synthetic 17-mer primers. (B) ORFs longer than 100 nucleotides and directions of transcription are shown for all six reading frames.
FIG. 2. Complete ribosome binding sequence of the hsp gene and upstream region. The putative promoter regions, −35 and −10, are underlined. A putative ribosome binding site (RBS) is boxed. The location of the 3' end of Tnf is also indicated. Sequence data have been deposited with GenBank under accession no. M30192.
be important in designing combinations in which unrelated but cooperative defense mechanisms can be genetically stacked.

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LITERATURE CITED


