

## 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 conjugative plasmid pTR2030, which completely inhibits plaque formation by small isometric phage (6). A prolate phage ( $\phi$ c2) 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 pCI750, 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<sup>+</sup> 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-mers) 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<sup>+</sup> (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<sup>+</sup> (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<sup>+</sup> 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<sup>+</sup> 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

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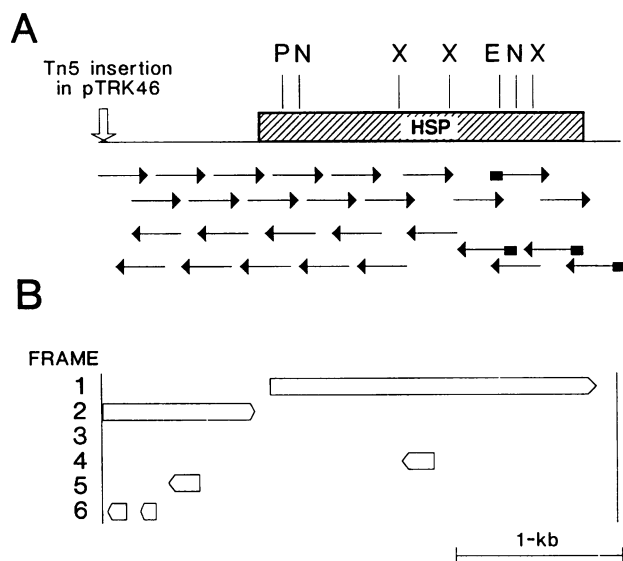


FIG. 1. Physical map and sequencing strategy for the 3.0-kb region from pTRK46 encoding Hsp<sup>+</sup> activity. (A) Physical map showing some of the restriction sites used to generate subclones for sequence analysis. E, *EcoRV*; X, *XbaI*; P, *PvuII*; N, *NcoI*. Symbols: →, direction and length of 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.

with the major ORF. This is supported by the fact that the presence of Tn5 in pTRK46 did not alter expression of the Hsp<sup>+</sup> 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 to 16 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 NPBRF [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 genotypically 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 pBlue-script (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 detected to 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 observed in ME2 and N1. 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<sup>+</sup>) 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

30 50
   
**ATA**GAGTCAGATGTGACCGTCATAATGATTACGAGGATAGCTGCAACGCTTG
   
 70 90
   
 GTTTCGAGTTAAATGTGCGTCAACGATTTGACGGAGAATTTGAAAAATTTCAAAGT
   
 130 150
   
 TCACGATGTTGACACTTACGATAAAAAAGAAAAATAGATTTTCATCGGAGATTGTC
   
 170 190 210
   
 TGATGCCTTGGTCCCAATAATATCTTCAGAAGATGTTGAATTTGAAGCOGAGCA
   
 230 250 270
   
 ATTTTTGAATTTGTATTTCCAGTAGCTATGGAATTCCTCAGOGTATTGACCC
   
 290 310
   
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 390 410 430
   
 AGAAATCAAGGCAAAAACTATCCTGATTGATCCTCGTGCATCGAAAGCOGTTG
   
 450 470
   
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 490 510 530
   
 TAGCGTTGCATTTGAATTTGGTTAGCGTTTTTCCAACCGAGCTTTTGAATATTAC
   
 550 570 590
   
 CACAACATAAGAAGAGTTGATGGGCTCATTGAGAAAAATATGACTCCACAGA
   
 610 630
   
 TTGGCTGGAGATTCAAGCTAGAAAAGATTACGCCATAAACTTGTATGCCAAGAA
   
 670 690
   
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 710 730 750
   
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 870 890 910
   
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 930 950 970
   
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 1170
   
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 R A V N M I K N I P P S A K N K Y F
   
 1210 1230
   
 CAACTTTTCTCTTTTGTGTAAGTGAACCCAGCTGGGAAGAGTTACTTTCT
   
 Q T F P F F L L S E T S W E E L L S
   
 1250 1270 1290
   
 GAAAATTTTTTCTACTCATATATAAGTCCAGGAGATTTCTGACITATCAAGAA
   
 E N F F Y S Y I K S G E F L T Y Q E
   
 1310 1330 1350
   
 AACCTTTTCTTACGATAGGACAAATTCAAAAGAGCCATGGAGCATAACGACAA
   
 N L S F Y D R T I Q K S H G A Y R Q
   
 1370 1390 1410 1430 1450
   
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 T R I V S P I I Y I F L I A I A S Q
   
 1470 1490 1510
   
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 V E R I Y V E K R T N D M S V Y F S
   
 1530 1550
   
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 G S F E K E K N T A H Y K Q S Y N T
   
 1570 1590 1610 1630 1650 1670
   
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 Y M T E L N A C Q E E F D Y Y F Q T
   
 1690 1710
   
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 D F S T F F H L V D T D N L F N K I
   
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 D R L D P K S A L V Y S S L I K M I
   
 1730 1750
   
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 G Q G R M P I V D G N S G L S F L N
   
 1750 1770
   
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 T V L Y L D D F D K E I I D S L K T
   
 1790 1810 1830
   
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 1850 1870 1890
   
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 F I K C A N K D L D F L N Y K V Y N
   
 1910
   
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 L L C E K A T K H H L E I N S S K T
   
 1950 1970 1990
   
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 K S F T P T S E L S T K M N T D L Y
   
 2010 2030 2050
   
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 N F F V Y N E D V D F E Q Y F S K N
   
 2070 2090
   
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 2110 2130 2150
   
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 2170 2190 2210
   
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 D G S Y I L N A I V Y N K S T W S Q
   
 2230 2250
   
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 2290 2310
   
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 2390 2410 2430
   
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 2450 2470
   
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 N H K D L M T I L K A D D H G I K E
   
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 2550 2570 2590
   
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 2870 2890 2910
   
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 S H S S A G L L Q N E D F S R Y R V
   
 2930 2950 2970
   
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 K S S L N D L K I I I E Q L S T L L
   
 2990 3010
   
 CAAAATAAAAACAGATTATAAATAATTTATTTCTGTTTTTATTTTTTCAAAAAGA
   
 Q N K N R L \*
   
 3030 3050 3070
   
 ATCAAAGTTTATAGATTTTTTCAACTGCTGTTATCTCTCCTCCTCCGCAATTTGG
   
 ATAAA

FIG. 2. Complete nucleotide 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 Tn5 is also indicated. Sequence data have been deposited with GenBank under accession no. M30192.



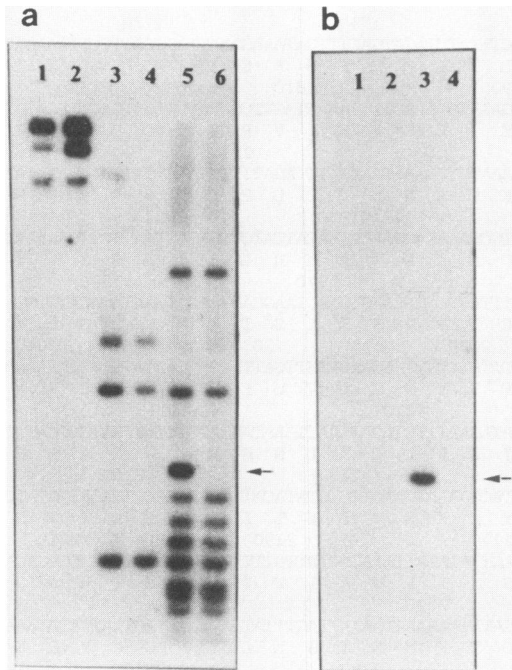


FIG. 3. Southern hybridization of lactococcal DNA with probes pTRK67 (a) and pTRK73 (b). (a) Lane 1, Uncut ME2 plasmids; lane 2, uncut N1 plasmids; lane 3, *Sau3A*-digested ME2 plasmids; lane 4, *Sau3A*-digested N1 plasmids; lane 5, *Sau3A*-digested ME2 total DNA; lane 6, *Sau3A*-digested N1 total DNA. (b) Lane 1, Uncut ME2 plasmids; lane 2, uncut N1 plasmids; lane 3, *Sau3A*-digested ME2 total DNA; lane 4, *Sau3A*-digested N1 total DNA. ←, Position of the 2.4-kb *Sau3A* fragment which contains the carboxy terminus of the *hsp* gene.

be important in designing combinations in which unrelated but cooperative defense mechanisms can be genetically stacked.

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