Journal of Applied Microbiology 2001, **91**, 334–343

Nucleotide sequence and characterization of the cell envelope proteinase plasmid in *Lactococcus lactis* subsp. *cremoris* HP

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714/1/01: received 10 January 2001, revised 14 March 2001 and accepted 4 April 2001

C. CHRISTENSSON, C.J. PILLIDGE, L.J.H. WARD AND P.W. O'TOOLE. 2001.

Aims: The major cell envelope proteinase (lactocepin; EC 3.4.21.96) produced by *Lactococcus lactis* cheese starter bacteria is required for starter growth and acid production in milk. The aim of this study was to characterize a lactocepin plasmid from a *L. lactis* subsp. *cremoris* cheese starter strain.

Methods and Results: A restriction map of the lactocepin plasmid pHP003 from strain HP was constructed, fragments were cloned in *Escherichia coli* vectors, and the complete DNA sequence (13 433 bp) was determined. Among 120 industrial *L. lactis* starter strains screened, five contained the same specificity-type lactocepin as pHP003. The lactocepin gene in these strains was invariably linked with a partially-deleted *abiB* gene.

Conclusions: The lactocepin specificity type of strain HP, conferred by a known configuration of key residues, is relatively uncommon. The gene is invariably linked with a partially deleted *abiB* gene on each lactocepin plasmid.

Significance and Impact of the Study: This is the first complete sequence reported for a lactocepin plasmid, and provides the basis for better understanding, or manipulation, of lactocepin production.

INTRODUCTION

Numerous amino acids are either essential or stimulatory for growth of *Lactococcus lactis* (Reiter and Oram 1962; Thomas and Pritchard 1987). In milk, most of these amino acids, available either as free amino acids or as low molecular weight peptides, are in short supply. Therefore, *L. lactis* strains used as starter cultures in cheese and lactic casein manufacture must be able to degrade casein present in milk in order to grow and produce acid (Christensen *et al.* 1999). The first step in the proteolytic system of *L. lactis* is the degradation of casein into shorter peptides by the cell envelope proteinase, for which the trivial name lactocepin (EC 3.4.21.96) has recently been proposed (Reid and Coolbear 1998).

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The lactocepin protein was first identified in *L. lactis* subsp. *cremoris* HP (Exterkate 1975) and subsequently, in other *L. lactis* strains. It was originally classified into different types according to the pH and temperature for optimal proteolytic activity (Exterkate 1976), and subsequently, by degradation patterns of α_{s1}-, β- and κ-casein (Visser *et al.* 1986). Strain HP produces a type I lactocepin which preferentially degrades β-casein and, to a lesser extent, α_{s1}-casein (Exterkate and de Veer 1985; Visser *et al.* 1986). More recently, lactocepins have been classified into groups based on their caseinolytic specificity for fragment 1–23 of α_{s1}-casein (Exterkate *et al.* 1993; Kunji *et al.* 1996).

To date, the gene for the lactocepin enzyme (*prtP*) has been cloned and sequenced from only four *L. lactis* strains (Kok *et al.* 1988; Kiwaki *et al.* 1989; Vos *et al.* 1989; Law *et al.* 1992). Related genes have also been cloned and sequenced from *Lactobacillus paracasei* subsp. *paracasei* (Holck and Næs 1992), *Lactobacillus delbrueckii* subsp. *bulgaricus* (Gilbert *et al.* 1996) and *Lactobacillus helveticus* (Pederson *et al.* 1999). Lactocepin genes studied to date are

all plasmid located in *L. lactis*; these plasmids range in size from 13·4 to 100 kbp (Kok 1990). Loss of these plasmids results in a lactocepin-negative (Prt$^-$) phenotype and slow growth of strains in milk (Pearce *et al.* 1974). Of the plasmids harbouring *prtP*, pUCL22 has been shown to be a theta replicator (Frère *et al.* 1993), and this mode of replication has been proposed for pWV05 (Seegers *et al.* 1994), based on sequence similarity to the theta replicating pWV02 (Kiewiet *et al.* 1993). *Lactococcus lactis* subsp. *cremoris* HP contains the smallest known (13·4 kbp) lactocepin plasmid (Larsen and McKay 1978; Kok 1990) and is a strain known historically to produce bitter-flavoured Cheddar cheese (Emmons *et al.* 1962; Lawrence and Gilles 1969).

In this study, the entire lactocepin plasmid (designated pHP003) has been sequenced from *L. lactis* subsp. *cremoris* HP, and the presence of lactocepins with identical specificity in five out of 120 industrial *L. lactis* starter strains has been demonstrated. The results show that the HP-type lactocepin is genetically linked with a partially-deleted, non-functional, phage abortive infection gene and a transposase gene.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, media and growth conditions

Lactococcus lactis strains were obtained from the New Zealand Dairy Research Institute culture collection and are listed in Table 1. *Escherichia coli* ER2206 [*endA1 thi1 supE44 mcr67* (*mrcA*) (*mrcBC-hsdRMS-mrr*)*114*::IS*10* (*lac*)*U169*/*F′ proAB lacI*^{q} *ZΔM15* Tn*10*] was a kind gift from New England Biolabs (Beverly, MA, USA). The lactococcal bacteriophages used (two prolate phages, c2 and 643, and eight small isometric phages, p2, sk1, 644, 712, 742, 748, 1766 and 5207) are described elsewhere (Pillidge *et al.* 2000).

Lactococci were grown at 30°C in M17 liquid medium (Terzaghi and Sandine 1975) or in 10% (w/v) reconstituted low heat skim milk powder (RSM). Media were supplemented with 10 g l^{-1} glucose for strains unable to ferment lactose. For solid media, agar was added to 1·5% (w/v). Transparent citrate milk agar (TCMA), used to screen proteolysis of strains, was prepared as previously described by Brown and Howe (1922) and Stadhouders (1961). *Lactococcus lactis* cultures on TCMA plates were incubated microaerobically at 25°C in a GasPak anaerobic jar (Becton Dickinson, MD, USA).

Escherichia coli ER2206 was grown at 37°C in LB medium (Sambrook *et al.* 1989). Transformants containing recombinant pUC19 plasmids were selected on plates containing 200 μg ml^{-1} ampicillin, 50 μg ml^{-1} X-gal and 1 mmol l^{-1} IPTG.

Molecular methods

Plasmid DNA of *L. lactis* subsp. *cremoris* strains was isolated as described previously (Anderson and McKay 1983) and then purified using a caesium chloride gradient centrifugation (Sambrook *et al.* 1989). *Lactococcus lactis* subsp. *cremoris* MG1363 was made electrocompetent by growth in the presence of glycine, and was transformed with the whole plasmid mixtures of HP or LW1484 by electroporation (Holo and Nes 1989). Strains HP and LW1484 grow too poorly in glycine to obtain enough cells for electroporation,

Table 1 *Lactococcus lactis* strains used in this work

Strain	Characteristics	Lactocepin plasmid size (kbp)	Reference or source
L. lactis subsp. *cremoris*			
HP	Industrial starter	13·4	NZDRI*
CJ	Industrial starter	13·4	NZDRI
FG2	Industrial starter	13·4	NZDRI
LW1431	Industrial starter	13·4	NZDRI
104	Industrial starter	30	NZDRI
LW1484	Industrial starter	34	NZDRI
HP Prt$^-$	Strain HP cured of its endogenous lactocepin plasmid		This work
HP/LW1484	Strain HP Prt$^-$ with the lactocepin plasmid from strain LW1484		This work
MG1363	Plasmid-free strain derived from *L. lactis* subsp. *cremoris* NCDO 712		Gasson (1983)
L. lactis subsp. *lactis* biovar diacetylactis DRC3			McKay and Baldwin (1984)

*NZDRI – New Zealand Dairy Research Institute culture collection.

so threonine was used instead (van der Lelie *et al.* 1988). The milk-clotting phenotype and plasmid profile of Prt^+ MG1363 transformants were checked to verify the presence of individual lactocepin plasmids.

Plasmid pHP003 was isolated from MG1363 (pHP003). A physical map of plasmid pHP003 was constructed by restriction with enzymes *Bam*H I, *Bgl* II, *Eco*R V and *Hin*d III (Boehringer Mannheim), and *Eco*N I, *Hin*c II and *Sap* I (New England Biolabs). Standard molecular genetic techniques were employed (Sambrook *et al.* 1989) for subcloning of fragments from pHP003 into the plasmid pUC19 (Yanisch-Perron *et al.* 1985), and for transformation of *E. coli* ER2206 with restriction fragments of pHP003 ligated into pUC19. Recombinant plasmids were prepared from *E. coli* using the CONCERT High Purity Plasmid Midiprep System (Life Technologies, Auckland, New Zealand).

To amplify by PCR a probe for the *prtP* gene, two custom primers were synthesized, PrtP1 5′-AAACACGGATGTCATCACTCAGG and PrtP3 5′-CCTTTAGCGTCAGCAGTATAGTC (Life Technologies), corresponding to positions 6645–6667 and (complement) 6836–6814 of the *prtP* sequence on pHP003, respectively. To amplify the *abiB* gene (Cluzel *et al.* 1991), primers AbiBL 5′-CTTTTGAGCAAGTTGATAGC and AbiBR 5′-TTCTTGGATAGGGGTAAG were used. Both products (192 bp and 705 bp, respectively) were amplified by Taq DNA polymerase (QIAGEN GmbH) using standard PCR conditions. The PCR products were purified, following agarose gel electrophoresis, with the QIAEX II Gel Extraction Kit (QIAGEN) and labelled with the ECL direct nucleic acid labelling and detection system (Amersham Pharmacia Biotech, Auckland, New Zealand). Southern blotting of target DNAs and DNA hybridizations using these PCR products was performed under stringent conditions, according to the manufacturer's recommendations.

DNA sequencing

Both strands of pHP003 were sequenced by primer walking, using custom primers (Life Technologies) designed from available sequence data. Double-stranded templates were sequenced using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Biosystems, Milton, Australia) and a MJResearch (Boston, MA, USA) PTC-100™ thermal cycler, and analysed with an ABI Prism 377 automated sequencer. To assemble the data, the Sequencing Project in the software GeneWorks® 2·5 (Oxford Molecular Group Inc., Campell, CA, USA) was used. Sequence data were analysed using GeneJockey (Biosoft, Cambridge, UK) and BLAST (Altschul *et al.* 1997) programmes. The sequence of pHP003 has been deposited in the GenBank Database under the accession number AF247159.

Construction of starter strain derivatives and analysis of their plasmid-associated phenotype

Lactococcus lactis subsp. *cremoris* strains HP and LW1484 were cured of their lactocepin plasmids by repeated subculture in unbuffered M17 (Sinha 1989). The cultures were plated onto TCMA plates to screen for Prt^+ and Prt^- colonies. Prt^- strains were transformed with lactocepin plasmids by electroporation to restore the Prt^+ phenotype. To make these strains electrocompetent, cells were grown in the presence of threonine. The presence or absence of a lactocepin plasmid in strain derivatives was confirmed by PCR, plasmid profile, Southern hybridization, and by testing for ability to clot RSM at 30°C.

To test for a phage resistance phenotype encoded by pHP003, phage lysates of two prolate phages and eight small isometric phages (Pillidge *et al.* 2000) were spotted on bacterial lawns of MG1363 and MG1363 (pHP003) cells in 3 ml soft M17 agar (0·45% (w/v) agar). Clearance zones (indicating host phage sensitivities) were compared after overnight incubation at 30°C.

To assay growth and lactocepin activity levels in RSM, strains were inoculated (1%) from an overnight RSM culture into 100 ml RSM and grown in triplicate at 30°C. During growth, samples were taken for determination of pH and colony-forming units (cfu) by plating dilutions onto M17 agar plates. To assay lactocepin activity, cells were grown to pH 5·0–5·2, 25% tri-sodium citrate added (3 ml 50 ml^{-1} culture) and the pH readjusted to 7·0. Cells were washed, harvested and resuspended in 5 ml 50 mmol l^{-1} Tris pH 7·5 and the cell dry weight determined as described by Coolbear *et al.* (1992). Three 40 μl aliquots were taken from each cell suspension and reacted separately with 20 μl fluorescein isothiocyanate (FITC)-labelled β-casein at 30°C, as described by Twining (1984). Blanks containing FITC-labelled β-casein only were also assayed. Lactocepin activity is expressed as relative fluorescence units (RFU) per milligram of dry weight of cells per hour (one RFU is equivalent to approximately 0·3 pmol β-casein hydrolysed, estimated by fluorescence analysis of FITC-labelled β-casein). During the assay, cell autolysis was monitored by measuring the combined release of two closely-related intracellular marker enzymes, tagatose 1,6-bisphosphate aldolase (EC 4.1.2.40) and fructose 1,6-bisphosphate aldolase (EC 4.1.2.13) using the assay procedure of Crow and Thomas (1982).

RESULTS

Genetic organization of pHP003

Sequence analysis showed that the lactocepin plasmid pHP003 in *L. lactis* subsp. *cremoris* HP is 13 433 bp in size

with a GC content of 40·0%, which is higher than the average overall GC content of 35·4–37·3% in *L. lactis* (Kilpper-Bälz *et al.* 1982; Bolotin *et al.* 1999). pHP003 contains six putative open reading frames (ORFs) of which the encoded proteins show a considerable homology with known proteins (Fig. 1). On the basis of sequence identities of greater than 98% with database entries, two of these are the *prtP* and *prtM* genes necessary for lactocepin expression. DNA sequence comparisons showed that three of these ORFs encode proteins that are likely to be involved in replication, and one ORF encodes a transposase. In addition to these complete ORFs, pHP003 harboured a partially-deleted *abiB* gene and a region with similarities to lactococcal transposases.

Lactocepin genes

The *prtP* and *prtM* genes in *L. lactis* subsp. *cremoris* HP were located at positions 5377–11085 and (complement) 5057–4158 on pHP003 (Fig. 1), and had GC contents of 47·0% and 41·9%, respectively. A similar arrangement for divergent transcription of *prtP* and *prtM* genes exists on other lactococcal plasmids (Kok 1990). The *prtP* gene in HP was 5709 bp long, encoding a predicted preproprotein of 1902 amino acids and processed lactocepin of 1715 amino acids anchored to the cell wall. The HP lactocepin was most similar to PrtP of *L. lactis* subsp. *cremoris* NCDO763 and *L. lactis* subsp. *cremoris* Wg2 (both 98·2% amino acid identity).

The *prtM* gene (900 bp) of pHP003 showed high levels of sequence identity (> 98%) with *prtM* genes of *L. lactis* subsp. *cremoris* NCDO763, Wg2 and SK11, and 98·3% identity to *prtM* on pVS2 in *Lact. paracasei* subsp. *paracasei* NCDO151 (Holck and Næs 1992). At the protein level, PrtM in strain HP had two unique amino acids compared with the PrtM in these four lactic acid bacteria strains (serine instead of alanine at position 169 and valine instead of alanine at position 249). There is currently uncertainty of the significance of these unique residues. In addition, PrtM in strain HP showed 28·8 and 26·8% identity, respectively, to two hypothetical proteins in *Streptococcus pyogenes*, and 29·1% identity to PrsA involved in the late stage of protein export in *Bacillus subtilis* (accession number P24327) (Jacobs *et al.* 1993).

A partially-deleted phage abortive infection gene

In *L. lactis*, the phage abortive infection mechanism AbiB prevents phage growth by degradation of phage transcripts (Parreira *et al.* 1996). Sequence comparison of an ORF of 501 bp at position 12748–13248 on pHP003 (Fig. 1) revealed 96·4% nucleotide and 92·2% amino acid identities with the phage abortive infection gene *abi416* (now termed *abiB*) in *L. lactis* subsp. *lactis* IL416 (Cluzel *et al.* 1991). When analysed by PCR using the primers AbiBL and AbiBR, the complete *abiB* gene gave a product of 705 bp. When pHP003 was similarly analysed, a product of 830 bp was unexpectedly found. Sequencing this PCR product showed that one of the primers, AbiBL, had misprimed upstream of an *abiB* gene that was partially deleted in the 5′ end, missing 250 bp compared with the normal *abiB*. (not

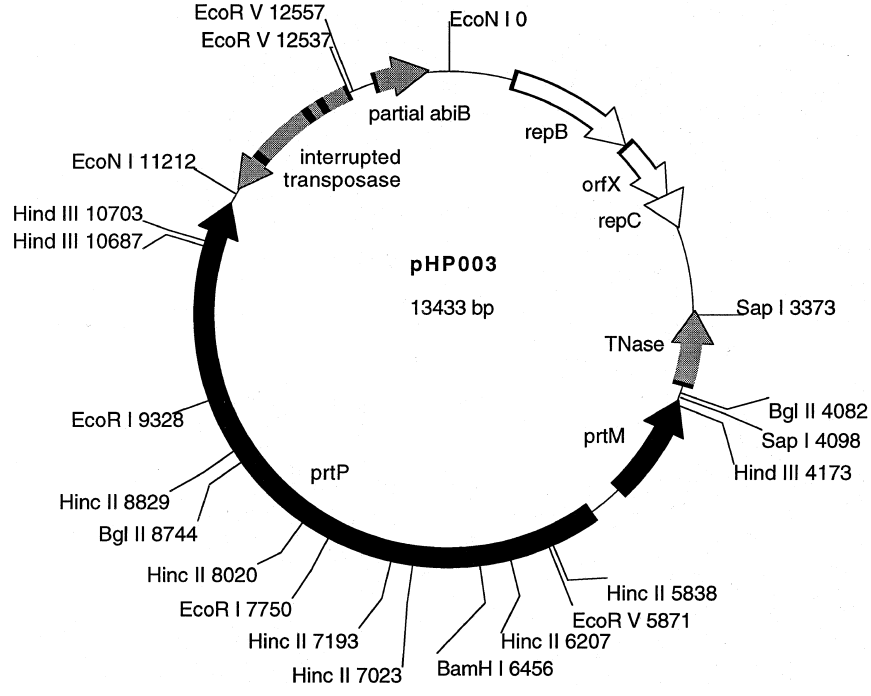


Fig. 1 Genetic map of pHP003 in *Lactococcus lactis* subsp. *cremoris* HP. Numbering starts at one of the *Eco*N I recognition sites just upstream of the origin of replication. The genes encoding PrtP and PrtM are depicted in black, the genes involved in plasmid replication (*repB*, *orfX*, and *repC*) are depicted in white, and the open reading frames for transposases (TNase) and the partially-deleted phage abortive infection protein are depicted in grey

shown). The foreshortened *abiB* gene on pHP003 had a low GC content of 26·4%. A sequence of 102 nucleotides immediately upstream of the partially-deleted *abiB* gene was identical to the *L. lactis* insertion sequence IS*981* (Polzin and McKay 1991), including the repeat sequence 5′-TAAAAACTTGACTTAACGTC. To investigate the possibility that the *abiB* gene had rearranged during recent laboratory passage or storage of the strain, a culture stock of *L. lactis* subsp. *cremoris* HP from 1954 was investigated by PCR using the same protocol as for the contemporary culture stock. The 1954 stock culture contained the same partially-deleted *abiB* gene. To confirm that the deleted gene did not somehow initiate from internal signals, phage sensitivity was tested. There was no detectable difference in phage sensitivity between the host *L. lactis* subsp. *cremoris* MG1363 and the recombinant MG1363 harbouring pHP003, when tested against two prolate phages and eight small isometric phages. Therefore, consistent with the *abiB* gene being partially deleted, pHP003 did not confer a phage resistance phenotype to any of the phages tested.

Replication and other genes

At co-ordinates 565–1722 on pHP003, *repB* was identified (Fig. 1), which encodes a predicted replication protein of 385 amino acids. The nucleotide sequence was 99·7% identical to *repB* in the plasmid pIL105 (accession number AF116286) from *L. lactis* IL964. In addition, the nucleotide sequence of *repB* in pHP003 was 78·7, 74·7 and 73·8% identical to the *repB* genes of the known lactococcal theta-replicating plasmids pUCL22 (Frère *et al.* 1993), pWV02 (Seegers *et al.* 1994) and pCI305 (Hayes *et al.* 1991), respectively. The putative start codon (ATG) of *orfX*, downstream of *repB*, overlapped with the end of the *repB* gene, suggesting that *repB* and a protein encoded by *orfX* may be translationally coupled. The sequence of *orfX* was 100% identical to the equivalent sequence on pIL105. Further downstream on pHP003, an ORF with 100% identity to an un-annotated ORF in pIL105 and *repC* in pND324 (accession number U44843) was identified.

Downstream of *prtM* on pHP003, there was an ORF from position (complement) 4008–3331 (Fig. 1) encoding a putative transposase of 226 amino acids. The sequence, including flanking inverted repeats, was 99·9% identical to the iso-IS*S1* transposase (TNase) located upstream of *abiD1* on pIL105 in *L. lactis* IL964 (Anba *et al.* 1995). The transposase in pHP003 had 93·2% amino acid identity to the sequence encoding a putative transposase of 236 amino acids upstream of *abiB* in *L. lactis* subsp. *lactis* IL416 (Cluzel *et al.* 1991). Compared with transposases found on other lactocepin plasmids, the transposase of pHP003 had 92·9, 92·0 and 85·8% amino acid identity (respectively) to the ORF-N1 and ORF-N2 proteins on pSK111 in *L. lactis* subsp. *cremoris* SK11 (Haandrikman *et al.* 1990; de Vos *et al.* 1991), and the ORF-W2 protein on pWV05 in *L. lactis* subsp. *cremoris* Wg2 (Haandrikman *et al.* 1990).

Plasmid-associated phenotype

The lactocepin genes of 120 industrial *L. lactis* starter strains were screened by PCR and DNA sequencing (L. Ward *et al.* manuscript in preparation). All strains produced a PCR product which was sequenced. Five *L. lactis* subsp. *cremoris* strains (CJ, FG2, LW1431, 104 and LW1484) were identified that contained identical HP-type I lactocepins. PCR and DNA sequencing of the products (not shown) indicated that the five strains also contained the same partially-deleted *abiB* gene found in pHP003. None of the other 115 starter strains, including those with different type-I lactocepins, contained the partially-deleted *abiB* gene.

The plasmid profiles of the industrial starter strains with the same lactocepin type as in strain HP are shown in Fig. 2a. All six strains appear different when analysed by pulse field gel electrophoresis (W. Kelly, personal communication). To identify the lactocepin plasmids, a Southern blot was performed using a *prtP*-probe (Fig. 2b). Strain HP, along with strains CJ, FG2 and LW1431, harboured the smallest lactocepin plasmid of 13·4 kbp. Strains 104 and LW1484 harboured medium-sized lactocepin plasmids of 30 and 34 kbp, respectively. The *abiB*-probe hybridized to the same plasmids in the six strains as the *prtP*-probe (Fig. 2c).

Growth and milk acidification rates, and lactocepin activities, were assessed in HP, LW1484, and an HP derivative in which pHP003 had been replaced with the medium-sized (34 kbp) lactocepin plasmid from LW1484. Analysis of the plasmid content of a derivative HP strain, cured of pHP003, showed that pHP003 was the only plasmid that was lost, and the *prtP* probe did not hybridize to any of the plasmids in the cured strain. The cured derivative, transformed with the 34 kbp plasmid from LW1484, only contained this plasmid in addition to its own plasmids, and the *prtP* probe hybridized only to the introduced 34 kbp plasmid (data not shown). The corresponding LW1484 derivative containing pHP003 could not be obtained, possibly due to the inability of LW1484 to form competent cells.

The growth and acidification rate in milk of the HP derivative was higher than both respective wild-type strains (Fig. 3a,b). However, wild-type HP with pHP003 had significantly higher lactocepin activity than the two strains harbouring the medium-sized lactocepin plasmid (Fig. 3c). During the lactocepin assay, release of intracellular enzymes with caseinolytic activity into the sample buffer could result in falsely-high readings. This was checked during the assay by measuring combined release of the intracellular marker enzymes tagatose 1,6-bisphosphate aldolase and fructose

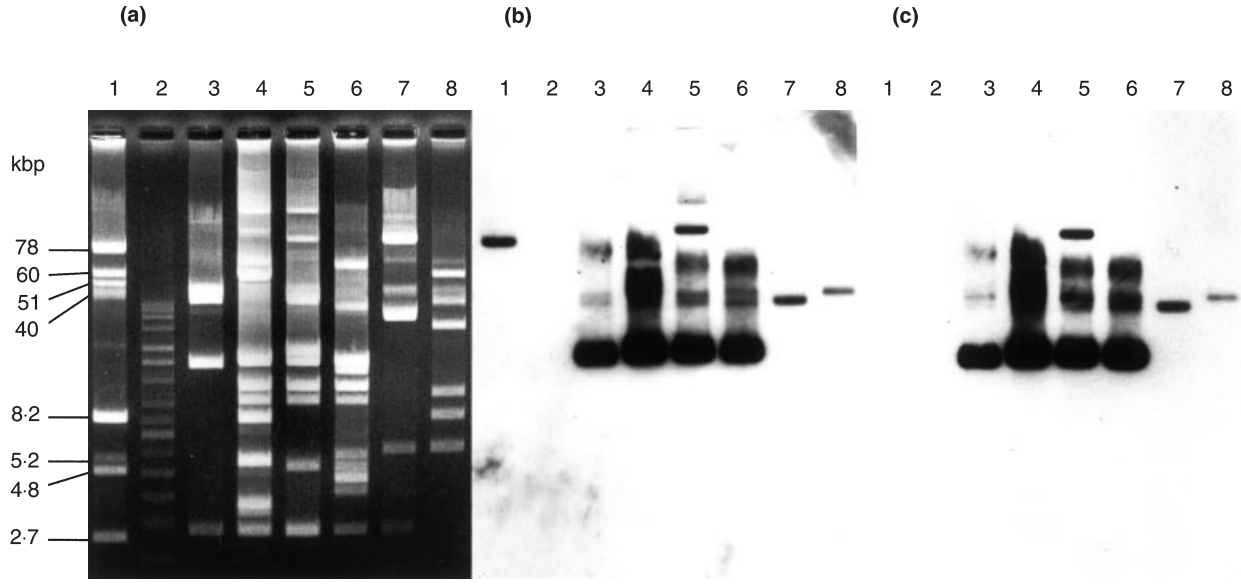


Fig. 2 Hybridization of labelled PCR products to total plasmids of industrial *Lactococcus lactis* strains. (a) Electrophoresis of total plasmids from *L. lactis* subsp. *lactis* biovar diacetylactis DRC3 (lane 1, size markers), Life Technologies supercoiled DNA ladder (lane 2), strains HP (lane 3), CJ (lane 4), FG2 (lane 5), LW1431 (lane 6), 104 (lane 7), and LW1484 (lane 8). (b) Southern blot with *prtP* as probe. (c) Southern blot with *abiB* as probe. The plasmid sizes from strain DRC3 in lane 1 are recalculated from the MDa reported previously (McKay and Baldwin 1984)

1,6-bisphosphate aldolase (Crow and Thomas 1982). It was found that the autolysis rate was not significantly different between strains (data not shown).

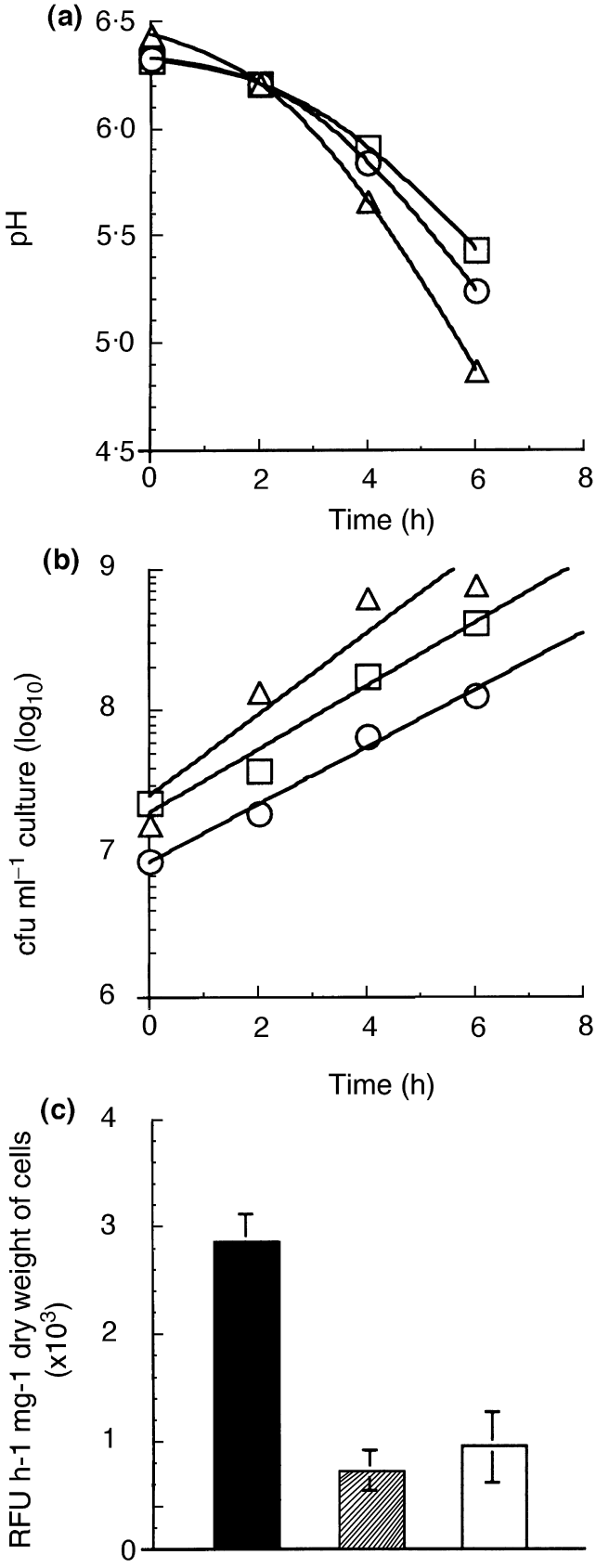
DISCUSSION

The plasmid pHP003 of *L. lactis* subsp. *cremoris* HP, first identified by Larsen and McKay (1978), is the smallest known lactocepin plasmid. As far as is known, this study presents the first complete DNA sequence of this plasmid or indeed, of any other lactocepin plasmid. It provides an opportunity to study a minimal replicon harbouring the lactocepin gene, which is indispensable in at least a proportion of cells for fast growth of *L. lactis* in milk.

Of the 120 industrial starter strains screened, five harboured type I lactocepins with identical specificity to that from *L. lactis* subsp *cremoris* HP, although their respective lactocepin plasmids differed in size. No lactocepin plasmid has previously been shown to harbour a gene for phage abortive infection. However, all six strains with the strain HP-type PrtP also had the partially-deleted *abiB* gene on their lactocepin plasmid, which suggests conservation of the genetic linkage. The same deletion was present in the *abiB* gene of a culture stock of strain HP from 1954. The sequence upstream of the partially-deleted *abiB* gene is similar to transposases, and the presence of IS elements suggests that a deletion may have occurred by rearrangement in the absence of selection for this Abi. The absence of phage pressure could have led by deletion to the production of a relatively small lactocepin plasmid in strain HP. However, it is notable that most lactocepin plasmids in *L. lactis* are in fact large. This suggests that they encode additional functions necessary for growth, other than the lactose fermentation pathway genes found on some lactocepin plasmids.

The specificity of the lactocepin type in strain HP is conferred by amino acids Thr-131, Thr-138, Asp-142, Leu-144, Asp-166, Leu-747 and Thr-748 (Exterkate *et al.* 1993). Among these positions, Asp-142 is unique, being found only in the HP-type lactocepin. In addition to this unique Asp residue at position 142, it was found that PrtP in *L. lactis* subsp. *cremoris* HP contained a further 16 unique amino acids compared with PrtP in other lactococci (Table 2). Of these 17 unique amino acids in *L. lactis* lactocepins, six were identical to residues in equivalent positions in PrtP in *Lact. paracasei* subsp. *paracasei* NCDO151 (Holck and Næs 1992). The conservative substitution of Ile for Leu at position 177 in the protease domain is unlikely to impact on proteinase specificity. However, the positively-charged Lys instead of the negatively-charged Glu at position 827 in the A-domain of other PrtP enzymes is more likely to affect the casein specificity of PrtP. Indeed, the region spanning residues 497–1089 has previously been shown to affect the casein specificity of PrtP hybrids from strains SK11 and Wg2 (Vos *et al.* 1991), though the PrtP molecules from both these strains have Glu at position 827.

The *prtP* gene of strain HP lacks the 180-nucleotide duplication found in the sequence encoding the cell-wall



◀

Fig. 3 Growth and proteolysis in milk. (a) pH decrease as a function of time. (b) Colony-forming units ml^{-1} culture as a function of time. (□), Strain HP, (△), recombinant strain HP with lactocepin plasmid from LW1484, and (○), strain LW1484. (c) Proteolysis as a function of dry weight of cells. Proteolysis is expressed as relative fluorescence units (RFU) mg^{-1} dry weight of cells h^{-1}, where one RFU is equivalent to 0·3 pmol casein, assuming there is a 1 : 1 stoichiometric relationship between FITC and β-casein. (■), strain HP, (▨), recombinant strain HP with lactocepin plasmid from LW1484, and (□), strain LW1484. Values graphed are the mean of three independent determinations, and error bars are the standard deviation about this mean

spanning domain of the SK11 lactocepin (Vos *et al.* 1989). The duplication is also absent in *prtP* of strain Wg2 (Kok *et al.* 1988) and strain NCDO763 (Kiwaki *et al.* 1989). The presence of this duplication in PrtP in some strains but not others may reflect differences in cell-wall structures between the strains. Surprisingly, the cell-wall spanning region of PrtP in strain HP is more similar to that of *Lact. paracasei* than the lactococcal proteinases.

Seegers *et al.* (1994) probed the *repB* gene of pWVO2 against a number of *Lactococcus* strains and strain HP hybridized very weakly. This is inconsistent with the 74·7% nucleotide identity between the genes. The presence of the *repB* homologue in the plasmid pHP003 is indicative of theta replication (Seegers 1994). Smaller lactococcal plasmids usually replicate by a rolling circle mechanism (Seegers 1994). Since pHP003 is probably a theta replicating plasmid, it probably arose by deletions occurring in a larger ancestral plasmid, although acquisition of *prt* genes by a smaller plasmid is also a possible mechanism.

Our data suggest that the size of the lactocepin plasmid may be important for determining the level of proteolytic activity, since the smaller plasmid of strain HP yielded higher proteolytic activity per milligram of dry weight of cells. This is most likely due to a higher copy number for the smaller plasmid and the gene dosage effect of *prtP* and *prtM*, but this has not been experimentally verified. Despite conferring higher proteolytic activity relative to the other plasmids, pHP003 did not confer enhanced growth rate or acid production. The medium-sized lactocepin plasmid from strain LW1484 may harbour genes whose products contribute to these properties. Despite the high level of proteolysis in strain HP, other cellular processes or components are likely to be growth-limiting.

The high level of bitterness associated with strain HP in cheese-making (Emmons *et al.* 1962; Lawrence and Gilles 1969) makes this now well characterized strain and plasmid unsuitable for many industrial applications. Biochemical and structural studies of PrtP from strain HP currently underway may lead to exploitation of pHP003 as a food-grade vector for expressing another lactocepin gene, or other genes with desirable industrial properties.

Table 2 Divergent amino acids in PrtP of *Lactococcus lactis* subsp. *cremoris* HP compared with full PrtP sequences published for other lactococci

Domain*	Position of amino acid in HP†	Amino acid in HP	Amino acid in Wg2/NCDO763/SK11‡
Signal peptide	−178	Phe	Ile
Pre-pro domain	–	–	–
Protease domain	142	Asp§	Ser/Ala/Ala
	177	Ile§,¶	Leu
A-domain	827	Lys	Glu
	839	Val	Ala
B-domain	931	Ser	Gly
	1091	Ile	Thr
	1118	Pro	Ser
	1129	Ser	Ala/Ala/Thr
	1261	Ala¶	Val/Val/Glu
Helical domain	1405	Ala¶	Glu
	1605	Asp	Ala
Cell-wall domain	1613	Asp¶	Gly
	1622	Ser	Gly
	1634	Thr¶	Ile
	1673	His¶	Leu
	1675	Thr	Ser
Anchor domain	–	–	–

*Domains as predicted by multiple sequence alignment, secondary structure prediction and database homology searching methods (Siezen 1999).
†Numbering starts at the proteinase domain.
‡If only one amino acid is listed, the lactocepins of strains Wg2 (Kok *et al.* 1988), NCDO763 (Kiwaki *et al.* 1989) and SK11 (Vos *et al.* 1989) have the same amino acid at that position.
§Identical to PrtP in the industrial starter strains CJ, FG2, LW1431 and LW1484.
¶Identical to PrtP in *Lactobacillus paracasei* subsp. *paracasei* NCDO151 (Holck and Næs 1992).

ACKNOWLEDGEMENTS

We thank Tim Coolbear for measuring lactocepin activity. C.C. was supported by scholarships from the Swedish Institute and Bengt Lundqvists minne, SE Banken, Sweden. This study was supported by the New Zealand Foundation for Research, Science and Technology.

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