Molecular characterization of the restriction endonuclease gene (*scrFIR*) associated with the *ScrFI* restriction/modification system from *Lactococcus lactis* subsp. *cremoris* UC503

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The nucleotide sequence of the chromosomally encoded type II *ScrFI* restriction/modification system from *Lactococcus lactis* subsp. *cremoris* UC503 was completed. The *ScrFI* restriction endonuclease (ENase) has previously been shown to specifically recognize 5' CCN GG 3' sites, cleaving after the second cytosine and the degenerate central base. The ENase gene (*scrFIR*; 862 bp) was located between, and co-directionally transcribed with, two formerly characterized 5-methylcytosine methyltransferase genes, which encode proteins that independently confer protection against *ScrFI* digestion. *scrFIR* codes for a protein of 272 amino acids with a predicted molecular mass of 31470 Da, which agrees favourably with a previously estimated molecular mass of 34 kDa for this enzyme. The deduced sequence of this protein did not show any significant homology with known protein sequences, including the isoschizomeric *SsoII* ENase from *Shigella sonnei*. The ENase gene was cloned and expressed in *Escherichia coli* and *Lactococcus*; however, no *in vivo* restriction of phage was observed, suggesting that expression of the ENase gene may be repressed, or that the appropriate expression signals may be absent in the cloned constructs. The ability of *ScrFI* to cleave non-canonically modified 5' CCN GG 3' sequences suggested that some *ScrFI* sites may require complex modifications to fully impair digestion by this enzyme.

Keywords: bacteriophage resistance, lactic acid bacteria, endonuclease, methyltransferase

INTRODUCTION

The mesophilic lactococci are important functional components in a variety of dairy fermentations, where they contribute to a number of necessary and desirable characteristics including product pH, flavour, texture and aroma. One of the factors governing the choice of the strains employed is their insensitivity to phage infection. Efforts to limit the potentially drastic consequences of phage infection have stimulated intensive

research aimed at understanding phage–host interactions. Genetic approaches are being increasingly used to dissect these relationships at a molecular level, with one of the primary goals being the development of super-immune strains which remain insensitive to a range of phages over prolonged production regimes. These studies have led to the identification of at least four types of natural phage resistance mechanisms in lactococci, including adsorption blocking, injection blocking, abortive infection, and restriction and modification (R/M) systems (for a recent review see Garvey *et al.*, 1995).

R/M systems are the most frequently encountered resistance mechanism in *Lactococcus*, where they are typically plasmid-encoded (Hill, 1993; Klaenhammer & Fitzgerald, 1994; Garvey *et al.*, 1995). The nucleotide sequences of three complete systems have been pub-

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Abbreviations: ENase, restriction endonuclease; 5mC, 5-methylcytosine; MTase, methyltransferase; R/M, restriction/modification.

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lished: *LlaI* (Hill *et al.*, 1991; O'Sullivan *et al.*, 1995), *LlaDCHI* (previously referred to as *LlaII*; Moineau *et al.*, 1995) and *LlaBI* (Nyengaard *et al.*, 1996). In addition, the sequences of two chromosomally encoded methyltransferases (MTases) associated with the *ScrFI* R/M system from *L. lactis* subsp. *cremoris* UC503 (previously designated *Streptococcus cremoris* F; Schleifer & Kilpper-Bälz, 1987) have also been reported (Davis *et al.*, 1993; Twomey *et al.*, 1993). As in other R/M systems, the deduced amino acid sequences of all the lactococcal MTases contain distinguishing motifs which simplify their identification from primary sequence data (Klimasauskas *et al.*, 1989; Pósfai *et al.*, 1989; Wilson, 1992; Noyer-Weidner & Trautner, 1993; Kumar *et al.*, 1994; Timiniskas *et al.*, 1995). In contrast, no universally conserved motif persists throughout the restriction endonuclease (ENase) sequences, which are more heterogeneous and generally only show significant primary sequence homology with related isoschizomers. This is illustrated by the *LlaDCHI* ENase, which recognizes 5' GATC 3' sites and shows significant identity with its isoschizomers *DpnII* (31%) and *MboI* (34%; Moineau *et al.*, 1995) but has relatively little homology with other ENases. While the majority of type II ENases do not have any obvious homology at the primary sequence level, X-ray crystallography studies suggest that many of these proteins may have similar tertiary structures, especially throughout the active-site residues, with more extensive structural homology among ENases that generate similar cleavage patterns (Anderson, 1993; Aggarwal, 1995).

Here the completed sequence of the *ScrFI* R/M locus is presented. The restriction gene (*scrFIR*) has been identified as being located between the two previously characterized MTase genes (Davis *et al.*, 1993; Twomey *et al.*, 1993). Unlike the cognate MTases, which show significant similarities with other 5-methylcytosine MTases, the deduced amino acid sequence of the *ScrFI* ENase is very different from other type II ENases, including isoschizomeric enzymes. The ability of *ScrFI* to cleave at its recognition site when some of the residues are methylated at the 5-carbon position is investigated.

METHODS

Bacterial strains, phage, plasmids and media. The bacterial strains, bacteriophage and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37 °C in LB agar (Sambrook *et al.*, 1989). Where necessary, the antibiotics ampicillin (Ap; 100 µg ml⁻¹), chloramphenicol (Cm; 25 µg ml⁻¹) or tetracycline (Tet; 10 µg ml⁻¹) were added to media. Lactococcal strains and their phage were routinely grown without shaking at 30 °C in M17 medium (Terzaghi & Sandine, 1975) containing either glucose or lactose as required (0.5%). Lactococcal cells containing pCI372 and various derivatives were selected and maintained by the addition of Cm (5 µg ml⁻¹).

Enzymes, molecular cloning and sequencing procedures. Restriction enzymes, modification MTases, T4 DNA ligase and Klenow fragment from DNA polymerase I were purchased from either New England Biolabs, Boehringer or Stratagene and used according to the manufacturers' instructions. Plas-

mid DNA was isolated from *E. coli* using the cleared lysate technique (Sambrook *et al.*, 1989) or by the method of Anderson & McKay (1983) for lactococcal DNA. Electrotransformation of DNA was accomplished using a Bio-Rad Gene Pulser apparatus and was performed according to manufacturer's instructions for *E. coli* cells or by the method described by Holo & Nes (1989) for *Lactococcus*. The nucleotide sequence of both strands of alkali-denatured double-stranded DNA was determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using a Sequenase II sequencing kit (United States Biochemical). Primers for sequencing were synthesized using an Applied Biosystems PCR-MATE DNA synthesizer.

Plasmid constructs and deletion derivatives. Reconstruction of the parental organization of the *ScrFI* R/M locus was accomplished by cloning an *EcoRI*/*Sau3A* fragment from pCI931m into the multiple cloning site of pBluescript KS I, followed by the introduction of an *EcoRI*-*NruI* fragment from pCI932m, generating a construct designated pCI933. The *E. coli*-*L. lactis* shuttle vector pCI372 was employed for the reintroduction of the *ScrFI* R/M chromosomal region into *L. lactis*. An *XhoI*-*Sau3A* fragment from pCI933 was introduced into the compatible *SalI*/*BamHI* sites within the multiple cloning site of pCI372, resulting in the formation of pCI934. A specific in-frame deletion of 582 bp from *scrFIR* was constructed by digesting pCI933 with *EcoRI* and *NdeI*, followed by filling in the recessed 3' ends with Klenow fragment from DNA polymerase I and religating. A construct designated pCI940, containing *scrFIBM*, *scrFIR* and *scrFIAM*, with a disrupted *orfX*, was constructed by cloning an *HaeIII*-*Sau3A* fragment into the multiple cloning site of pCI372.

Assay for ENase activity. *In vitro* biochemical assays and plaque assays were used to assess constructs for ENase activity. Overnight cultures of *E. coli* and *L. lactis* were pelleted by centrifugation, washed in quarter-strength Ringer's solution and resuspended in 0.75 ml ice-cold extraction buffer: 0.05 M Tris/HCl pH 7.5, 1 mM Na₂EDTA and 7 mM 2-mercaptoethanol. Cells were ruptured by adding 0.5 g glass beads (106 µm and finer; Sigma) and vibrated for 7 min using a 'Shake it Baby' cell disrupter (Biospec Products). Cellular debris was removed by centrifugation (approx. 14000 g, 10 min) and a 10 µl aliquot of the cell-free extract was analysed for its ability to digest 1 µg pBR322 DNA in a 40 µl reaction mixture containing 10 mM Tris/HCl pH 7.5, 5 mM MgCl₂, 100 mM NaCl, 5 mM dithiothreitol and 2 mM 2-mercaptoethanol. Reactions were incubated at 37 °C for 60 min followed by electrophoresis. Biological *ScrFI* activity was assessed by comparing the titres of the lactococcal phage ϕ c2 on the host of interest relative to a non-restricting host. Enumeration of phage was performed by mixing 1 ml of the appropriate phage dilution with 0.1 ml of an overnight culture of *L. lactis*, adding the mixture to 3 ml prewarmed (45 °C) soft agar (0.75%) supplemented with 2 mM CaCl₂ and pouring onto prepour agar. The efficiency of plaquing (EOP) of bacteriophage was defined as (phage titre on the host of interest)/(phage titre on a non-restricting host).

Assay for activity of *ScrFI* on 5-methylcytosine-modified DNA. The effect of non-canonical 5-methylcytosine (5mC) modifications on impairing DNA cleavage by the *ScrFI* ENase was assayed using *in vitro*-methylated DNA. pBR322 DNA was isolated from a *dcm*⁺ *E. coli* strain (RR1) and 1 µg was methylated *in vitro* with 6 U of *M.SssI* or *M.MspI* in the presence of 80 µM S-adenosyl-L-methionine for at least 4 h in the appropriate buffer as recommended by the manufacturer (New England Biolabs). One MTase unit was defined as the

Table 1. Bacteria, bacteriophage and plasmids

Relevant genotype or phenotype		Reference or source
Bacteria		
<i>E. coli</i>	$\Delta(mcrA)183 \Delta(mcrBC-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1$	Stratagene
XL1-Blue MRF	$gyrA96 relA1 [F' proAB lacI^q \Delta M15 Tn10 (Tet^r)]$	
RR1	$F^- \Delta(gpt-proA)62 leu supE44 ara-14 galK2 lacY1 \Delta(mcrC-mrr) rpsL20(Str^r) xyl-5 mtl-1 recA^+$	Sambrook <i>et al.</i> (1989)
<i>L. lactis</i> subsp. <i>cremoris</i>		
MG1363	Plamid-free derivative of <i>L. lactis</i> subsp. <i>cremoris</i> 712	Gasson (1983)
UC503	Isolate from Irish Cheddar cheese mixed-strain starter encoding <i>ScrFI</i> R/M activity	Fitzgerald <i>et al.</i> (1982) Costello (1988)
UC563	Derivative of <i>L. lactis</i> subsp. <i>cremoris</i> UC503 cured of the phage-resistance adsorption-blocking plasmid pCI528	
Bacteriophage		
$\phi c2$	Prolate-headed lactococcal phage susceptible to <i>ScrFI</i> restriction	UCC Phage Collection
Plasmids		
pCI372	5.7 kb <i>E. coli</i> - <i>L. lactis</i> shuttle vector, Cm^r	Hayes <i>et al.</i> (1990)
pCI931m	pBR322-based chimeric construct containing <i>scrFIAM</i> modification gene from <i>L. lactis</i> subsp. <i>cremoris</i> UC503, Ap^r	Davis <i>et al.</i> (1993)
pCI932m	pBR322-based chimeric construct containing <i>scrFIBM</i> modification gene from <i>L. lactis</i> subsp. <i>cremoris</i> UC503, Ap^r	Davis <i>et al.</i> (1993); Twomey (1996)
pCI933	pBluescript-based construct with a 4.2 kb <i>NruI</i> - <i>Sau3A</i> fragment from <i>L. lactis</i> subsp. <i>cremoris</i> UC503 containing <i>orfX</i> , <i>scrFIBM</i> , <i>scrFIR</i> and <i>scrFIAM</i> , Ap^r	This study
pCI934	pCI372-based construct with 4.2 kb <i>XhoI</i> - <i>Sau3A</i> fragment from pCI933 containing <i>orfX</i> , <i>scrFIBM</i> , <i>scrFIR</i> and <i>scrFIAM</i> , Cm^r	This study
pCI940	pCI372-based construct with a 3.8 kb <i>HaeIII</i> - <i>Sau3A</i> fragment from pCI933 containing <i>scrFIBM</i> , <i>scrFIR</i> and <i>scrFIAM</i> , Cm^r	This study
pCI942	Derivative of pCI933 with an <i>EcoRI</i> - <i>NdeI</i> deletion internal to <i>scrFIR</i> , Ap^r	This study
pBluescript KS I	<i>E. coli</i> cloning vector, Ap^r	Stratagene
pBR322	<i>E. coli</i> cloning vector, Ap^r Tet^r	Bolivar <i>et al.</i> (1977)

amount of enzyme required to fully protect 1 μ g λ DNA in 1 h at 37 °C in a 10 μ l reaction mixture against digestion by an appropriate ENase. To demonstrate complete modification, the *in vitro*-methylated plasmid DNA was treated with 5 U of either *HpaII* or *MspI* for 1 h, where appropriate, and examined for products of ENase activity using agarose gel electrophoresis. Incubation of fully modified DNA with *ScrFI* was used to assess the effect of non-canonical 5mC modifications on cleavage by this ENase. One ENase unit was defined as the amount of enzyme able to digest 1 μ g of λ DNA at 37 °C in 1 h.

Sequence data analysis. This was performed using the DNASTAR software and the facilities available at the Irish National Centre for BioInformatics (INCBI, Trinity College, Dublin, Ireland).

RESULTS

Sequencing and phenotypic analysis of the *ScrFI* R/M system

L. lactis subsp. *cremoris* UC503 was isolated from a mixed-strain starter culture employed in the Irish Cheddar cheese industry and was previously shown to

encode a type II restriction ENase, *ScrFI*, which recognizes the sequence 5' CC⁺NGG 3' and cleaves as indicated (Fitzgerald *et al.*, 1982). Two constructs, pCI931m and pCI932m, were isolated from the chromosome of this strain; each encoded a 5mC MTase which conferred resistance to *ScrFI* digestion (Davis *et al.*, 1993; Twomey *et al.*, 1993). Inverse PCR analyses coupled to Southern hybridization suggested that the two *EcoRI*-*Sau3A* fragments from these MTase-encoding constructs were adjacent to each other on the lactococcal chromosome, sharing a common *EcoRI* site. To confirm this organization and to exclude the possibility of two closely associated *EcoRI* sites occurring at this location, this region of the chromosome was amplified by PCR and sequenced directly. In agreement with the earlier work of Davis *et al.* (1993), this analysis indicated that the inserts of pCI931m and pCI932m are indeed contiguous on the UC503 chromosome, sharing an *EcoRI* site (Twomey, 1996).

The sequences of two 5mC MTase genes from pCI931m and pCI932m have been previously reported (Davis *et al.*, 1993; Twomey *et al.*, 1993). The sequences of the

TAAATATTACACTTGAAACACAAAGATCTGGTGAACGTCTGTACCTTACTCAAAAGAACAAACGCAACACTCCAGACAAATGGAACCTAAAAAATCTCTAAAAAATTCGTAACACGTAATCTTTAAAGAAGTTAA 140

TAAGGTTTCATTACATGTCAATAGACTTGTAAAAATTGATTTAGAGCGATTCTTGAATTTGCAATTTAGCTTCAACCCGATGACTACATTTTGACTACATTCTTGAATATAAAATCTTTGGG 280

-10 **-35** **RBS** **oriX** → M T P I M S F W P S I Y D K I K N Q I K L I E Y R R T

TTTCCCTAACGACTGTAATATGCTTATATGATTAATTAACCCGTTAAAGCCATTGGTGGTATTTGTTTTCGAAAAAACATGATTTAGAGGACTGAAAAAGCAATATTCAATAATACCATTTCTCTGACC 580

F P N D C K Y A Y M Y I T K P V K A I G G I Y Y F G K K H D L D D W K K Q Y S N N T I I S D

GCATTAATAGTTATATGAGTATCGTTACGGAATGGAATTAATGGTTTTCAGAAAAATCAATCTTACCTTGATGAGTACGAAAAATGTTGAAGGTTTCAGTGCACCAATCATATCTTTTACTTGAAGAAC 700

R I N S Y I Q S Y I G M E I I G F Q K I N P I T L D E L R K N V E G F T A P Q S Y L L L E N

AATAAAAAATTATCAGATTATGGAAGAACACACTGTTAACTCGGTCTTTTATGAAAAATGATTAATAATTTTCCCGAACATATATGCAAGGTTTAACTATGCTAGAGTATTTGAAGCATTCGACGG 840

N K K L S D Y V K N N T V K L G S F I E N D L S N I F P E H I C K R Y stop

CTACGAGTACGCTGACCTAATTAAGCAAAATTTCCACATGAAATTTCCGAAATTCGAGGAGATGACTGTTATCTTATTCGCAATTCATGAAATTTTACTTGAGGACGTAATAAAACATCA 980

Y G S Q R L A L I K A N I P H E I V G I S E J E G D V L L S Y S A I H E N L L E E R N K N I

AGCTAACAGATGATGAAATGCGTACTATCTCAAGAACATCAATTTCTTTAGATTATAAACTTTTGAATTCGAGCAGATAAGCTTGAGGTCAGAAATGAAAGATATGATATCGCAATAAATCAATAAAAT 1120

K L T D D E M R D Y L K N I N I P L D Y K T F E N R A D K L G G Q K L K D M Y I A N K L N K N

TTTGGTACATCCGCTCTATGACCCAAAAAATTTACCTGATTTTGACTTTTCACTTATCTTCCATGCCAAGATTTTGGTGTCTGGATATCAAAATGGATTAGTACAGATTTTGGACGAGGCTTCTCTATT 1280

F G D I R S I D P K K L P D F D F T Y S F P C Q D I A S V A G Y G L V A D S G T R S S L L

ATGGGAATGCTCAAAATATTGAGCATAAAAACCTTAAATTAATGATGAAAAATTTAAATAATTTAGTAGTAAAAATCATAAGTTAACTTCAATAATTTTATTACTAGATCTAGGATATACTAAT 1400

W E C C K I I E H K K P K Y L M H E N V K N L V G K N H K V N F N K F L L Y L E S L G Y T N

ATTGGGATTTCTCAATCTAGGACTTTGGTATCCCTCAAAATAGAGAACGAGTTTGTATCAGTATTTAAATCCTTAATGAGATTCTTACTTTCCCAAGCAAAATTTAACTTATCTATGAATGATTATTA 1540

Y W D I L N A R D F G I P Q N R E R V F C I S I L N P N E D F T F P Q K Q N L T L S M N D L L

GAGGAAATGTTCCGAAAAATTTTATCTCAAAATAACCAAGTACGACGACCACTTCTCAAGATTATATGTTTATGATTCAAAATTTGGAAGGAACTTTTAAAGATTTTTATCTAAATAAACGACG 1680

E E N V S E K F Y L K N N Q V S D E P I L Q D Y I Y C L D S N Y W K G T F L D S L K K R R

ACAATTAGTATCTGAAAAATGTCGCTGATGAAAAATCTCTGATAGAGACTTCTCCAGTGAACACTGGCGCTTCAAGGAGTAGAGGACTAATTTTGACAAAGCTAGTATCTCTGTAAGCAATACAGCTAT 1820

Q L V S G K V R P D G K Y P A R R L T P R E T W R F M G V E D T N F D K A S I L V S N T S L Y

ATAACAGTCTGAAATAGCATTGTTGTTCTGATTTGAATCGCTATTTAAGGAACATTTCAATACCAATAGCATTATTAAGTACCAAAATGAAAGCAAAATGAAAGGATTATCGAAATTTTGAAGTAAATGA 1960

K Q S G N S I V V P V L E S L F K E L F K S Q stop

RBS **scrFIR** → M N P N K E R L I E I F R S N V

AAAGGAAATTTCTCATTTTCAAGGAAATATACGCGATGCGGAGATGGGGCACTGTTAGAGAACGATTGGAATATCAGCAACGCTGACAACTGCGGATATTTGGCTATGAGTTAAATAAGGAGC 2100

X G R I P D I S G R N I R H D G R W G H W L E E R F G I S A N A D N H A D I L G Y E L K N E A

CACCTCTGGTAAACTACTTTTGGAGATTGGTCTGCAAAATGAATATATTTTAAACACCTCCTTATACTCTCTCTTTTCAGGCTCTACTGCTTCTGAGAGCAGAAATGCTTTTGTGCAATGTTGCGAAAGCCTAACG 2240

T S G K T T F G D W S A N E Y I F K T P P Y N S L F S G S T A S E K Q N A F C R H F G K P N

AAGCTAAAAATGGTGGTTCTCGTCTGGTTCTCTCTATTCCTCAAAATTTGGCAATATAATCTTTTGGTCAAAATTTGGTAATTTGAAGAAATTTGGATATTGTCATTATTTATTTCAATTAGTCAAGATTACGATAT 2380

E A K N G R F S W S G S P I P K I W Q Y N S F G Q I M V I E E N L D I V I Y Y S F S Q D L R Y

AATAGTTTGAATTTATACCCCACTTCAACATGATGAAATGCAATAGCAGCTTGGTATGGAGTTGCAAACTCTGTTAAGTCTAGAGGAAAACTCTTAAAGATAAACTAGAGATAAATTAATGACCTAGG 2520

N K F E I I P P Q L Q H D E I Q I A R W Y G V A N P L L S R R G K T L K D K L E D K F N D L G

TTGGTTACATGATCAACGATTCATAGGCGCATATGATAAAATTTGTTTGAAGCAATCACATTTGAAACTGGAATTAATCTAGTATGCTGCGCTTGTATTTTATAGTGGTATGATGAGGAAACAGC 2660

W F T C T T D S I G A Y D K I C F G R P I T F E N W I N L V D S G I V Y F G L V S N Y S

GCCCCATTCTCAGTGGAGCTGATAATAGCTACTGGAATAGTTAATTACGATGTCACCAATAAGAAATATGAGCATCTAATAAAAAATTAATTTTGAACGATATTTATCTGCTCTTTTCTTAAATAAAAT 2800

R P Y S Q W R A D N S Y W N S L I T D C H Q stop

AATCTTGCATTTCAATTGATTTATGTTATGTTAGTGTCAATCAGAGCAAAAAAGTCCTTATAAAACACCTTTATGCAAAAGCTTTATATGACTACTATATCAAGAAATACAGGAAACAAATTTCTATAATGATT 2940

-10 **-35** **RBS** **scrFIAM** → M T T I S R N T G T E I S I M I

AAAGAAAGGCACTACGCTAAATATGACTCAAAAGAGCTTCAGATGCAAGTGGGCACTGCAAAAGCGAGATGAAACCAATAAGAGATGCGAAATGGTGAACCTTGTCCATCAGAGCTTGAATATCTGCTATTTT 3080

K E K R L R L N M T Q K E L A D A V G S K N G D R T I R R W E N G E T C P S Q L E I S A I L

AAGATTTCCAGAAATGCAACCTTGAATAAGAAACAGCAATATAAATGATGACCTATTTGAGGATTTGAGGAGCAGCTTAGGTTTTCATCAACAGAAAAAGTATGCTGTATTTTCACTGAAATG 3220

R F P E I A P F E N R K T A K Y K M I D L F A G I G G V L I E Q K N K N P R K I T P R E A R

ATAAATTTGCTATTAATACTTATAAGCGAATTTTGGAGACGAACCTCATGGGATATAACAAAAATGATGAGAAAGATATCCAGACCATGATATCTTGTGCGGGATTTCTTGTCAAGCATTTAGTCAAGCAGGA 3360

D K F A I K T Y K A N F G D E P H G D I T K I D E K D I P D H D I L V G G F P C Q A F S Q A G

AAAAATTAGGATTTGATGATACGTTGAACTTTATTTTGAATAGCAAGAAATATAAAGAAAAACGCCATAAGCATTTCTACTGAAATGTAATAAATTTAAAAACCATGATAGAGGAAAGCTTTTAAAC 3500

K A L G F D D T R G T L F F E I A R I I K E K R P K A F L L E N V K N L K T H D K G R T F K T

AATATTAATACCTTTGGAAGAACTTGATTAAGTTACACAGCGCTATTCAGGCAAGAGATTTGGCTCCCAAAATAGAGAAAGGATTATATTGAGGTTTGAAGAAATCAATAGCAACTATCTGAT 3640

I L N T L E E L D Y E V H T A L F K A R D F G L C P Q N N R E R I Y I V G F D R K S I S N Y S O F

TTCAATGCTACACCTTTACAGAAAAAAGCTCGTGTGAAATATTTTGAATCGGTGTTGATGATAAATACAAATACAGATAAATTTGGGATGGGCATCAAGAGAAAAAGCTGAAAAATAAAAAATGGCAAA 3780

Q M P T P L Q E K T R V G N I L E S V V D D K Y T I S D K L W D G H Q R R K T E N K K N G K

GGTTTGGATATACCTCTTTAATCAAGATGAGTATACAAACACTTTATCAGCTGTTATTAAGATGGTATGAGTAACTAATTTGAACAGAAAAATAAAAATCCTCGAAAAATTTACTCTAGGGAAGCTGCTCG 3920

G F G Y T L F N Q D S E Y T N T L S A R Y K D G S E I L I E Q K N K N P R K I T P R E A R

ATTACAGGTTTTCAGAAAAATTTATTAATCTGTTAGCGATACCAAGCTTTATAAGAAATTTGAAACTGTTTGCAGTTCACCACCTCATGCTATTCAGAGAGAGATGTTGGAAGTTTGAAGAGCTCAAAAAAT 4060

L Q G F P E N F I I P V S D T Q A Y K E F G N S V A V P T I H A I A E K M L E V L E K S K K stop

AACTAAAAATCTCAGGTAATCTTATCATGAGATTTTATATTTCTCAAACTCTCTTTCTGTACTATTACGATATTAGACGATTTTGAACAGAGGAATAGTTGATCTCCCTTATTTCTCATCTGTTAGA 4200

BanI
TTATATCTTTGCTATCAATTTGAATCATCTACCATTCATTTTATCTTACGACACC4259

Fig. 1. Completed nucleotide sequence of the *ScrFI* R/M system and deduced amino acid sequences encoded by *oriX*, *scrFIBM*, *scrFIR* and *scrFIAM*. The sequences of two 5mC MTase genes, *scrFIBM* (Twomey *et al.*, 1993; GenBank accession no. L12227) and *scrFIAM* (Davis *et al.*, 1993; GenBank accession no. M87289) have been reported previously. The direction of transcription is illustrated by short arrows following the gene designations. Sequences equivalent to *E. coli* -10 and -35 promoter sequences are boxed. Predicted ribosome-binding sites are denoted RBS and underlined. Facing arrows indicate sequences likely to form significant secondary structures. Restriction sites used in cloning and in the formation of a number of plasmid constructs are also indicated.

Fig. 2. Schematic representation of the lactococcal inserts contained in plasmid constructs used in this study. The arrows represent ORFs and a stippled triangle illustrates a deleted fragment. Regions containing sequences consistent with lactococcal promoter sequences are marked P. Sequences likely to form significant secondary structures are depicted by a graphical representation of the predicted structure. Appropriate restriction sites used in cloning and mutational analyses are also indicated. The R⁺/R⁻ phenotype designations refer to ENase assays of cell-free extracts.

remainder of the chromosomal inserts of these plasmids, spanning a region of 6045 bp in total, were determined. PCR analysis indicated that fragments flanking a *Sau3A* site in this region (Fig. 1, nucleotide 4172) were contiguous on the UC503 genome, indicating that this site, which lies immediately downstream of *scrFIAM*, was not cleaved by the partial *Sau3A* digest used in the original cloning of the *ScrFI* MTases. In addition to the previously described ORFs (*scrFIAM*, *scrFIBM* and *orfX*; Davis *et al.*, 1993; Twomey *et al.*, 1993), analysis of other putative ORFs in this region identified one initiating 19 nucleotides downstream of the translational stop codon of *scrFIBM* and spanning the *EcoRI* site common to both inserts (Figs 1 and 2). This ORF is preceded by an appropriately spaced ribosome-binding site which bears complementarity to the 3' end of *L. lactis* 16S rRNA and has a predicted free energy of $-16.2 \text{ kcal mol}^{-1}$ ($-67.8 \text{ kJ mol}^{-1}$) at 25 °C. Furthermore, the deduced protein had a predicted molecular mass (31470 Da) in good agreement with the previously estimated molecular mass (34 kDa) of a partially purified preparation of *ScrFI* (Davis, 1992).

As the genetic loci responsible for encoding ENase and MTase activities in type II systems are invariably relatively close to each other, with intervening distances between the respective genes rarely greater than 0.7 kb (Wilson & Murray, 1991), the ORF between the two *ScrFI* MTase genes was investigated to determine if it

encoded the *ScrFI* ENase. A two-step cloning strategy was employed to reconstruct the parental organization of this putative ORF in which a 2.2 kb *EcoRI*–*Sau3A* fragment from pCI931m was first cloned into pBluescript KS I, followed by the introduction of a 2.0 kb *NruI*–*EcoRI* fragment from pCI932m (Fig. 2). This construct, designated pCI933, was electrotransformed into *E. coli* XL1-Blue MRF' and cell-free extracts of the transformants were examined for *in vitro* *ScrFI* activity (Fig. 3, lane 6). Incubation with pBR322 (Dcm⁻) clearly indicated type II ENase activity, which was consistent with similar activity observed in the parent *ScrFI*-producing strain, *L. lactis* subsp. *cremoris* UC503, and which was absent in the plasmid-free *E. coli* XL1-Blue MRF' (Fig. 3, lanes 3 and 5, respectively). Furthermore, an in-frame deletion mutant, in which an *EcoRI*–*NdeI* fragment internal to the ORF of interest was removed from pCI933, accounted for the loss of ENase activity (Fig. 2; Fig. 3, lane 7). As the *ColEI* origin of replication of pCI933 is unable to replicate in *L. lactis*, the shuttle vector pCI372, which contains the replication regions of plasmids pCI305 (isolated from *L. lactis* subsp. *lactis* UC317) and pBR322, was employed to reintroduce the fragment of interest into *L. lactis*. An *XhoI*–*Sau3A* fragment from pCI933 was subcloned into the compatible *SalI*/*Bam*HI sites within the multiple cloning site of pCI372 (generating pCI934), and transformed into plasmid-free *L. lactis* subsp. *cremoris* MG1363. Analysis of cell-free extracts from transformants harbouring the

Fig. 3. Analysis of various *E. coli* and *L. lactis* cell-free extracts for *ScrFI* ENase activity. Lanes 3–7, pBR322 DNA treated with CFE from *L. lactis* subsp. *cremoris* UC503 (lane 3); *L. lactis* subsp. *cremoris* MG1363(pCI934) (lane 4); *E. coli* XL1-Blue MRF' (lane 5); *E. coli* XL1-Blue MRF'(pCI933) (lane 6); *E. coli* XL1-Blue MRF'(pCI942) (lane 7). Lane 1, size markers (λ DNA digested with *Bst*EII); lane 2, pBR322 DNA (uncut); lane 8, pBR322 DNA incubated with commercially purified *ScrFI*.

pCI934 construct exhibited ENase activity, although the activity was poor relative to *L. lactis* subsp. *cremoris* UC503 and *E. coli* XL1-Blue MRF' harbouring pCI933 (Fig. 3, lanes 4, 3 and 6, respectively). This analysis indicated that the ORF residing between the two *ScrFI* MTase genes encoded the *ScrFI* ENase; consistent with the proposed nomenclature for genes associated with R/M systems (Szybalski *et al.*, 1988), this gene was designated *scrFIR*.

When *L. lactis* subsp. *cremoris* MG1363 harbouring pCI934 was examined for *in vivo* restriction of the prolate-headed lactococcal phage ϕ c2, no significant restriction was observed (Table 2). In contrast, substantial restriction was observed with *L. lactis* subsp. *cremoris* UC563, a derivative of the parent *ScrFI*-producing strain which has been cured of the cell-surface-exopolysaccharide-encoding plasmid pCI528, which masks biological R/M activity in UC503 (Table 2; Costello, 1988; Lucey *et al.*, 1992; Davis *et al.*, 1993). Evidence that the *ScrFI* R/M system contributed to the 10^3 -fold reduction in EOP observed, rather than an alternative R/M system encoded by UC563 being wholly responsible, was provided by purifying ϕ c2 which had been passaged through MG1363(pCI934) (containing both *scrFIAM* and *scrFIBM*) and re infecting the *ScrFI*-proficient host, UC563. Phage recovered from MG1363 (pCI934) were methylated by the *ScrFI* MTase(s) and

Table 2. Assessment of lactococcal strains for biological *ScrFI* R/M activity

ϕ c2.MG1363, ϕ c2.UC563 and ϕ c2.pCI934 refer to ϕ c2 recovered from single-plaque isolates following infection of *L. lactis* subsp. *cremoris* MG1363, UC563 and MG1363(pCI934), respectively. EOP data are expressed as the ability of ϕ c2 to infect the strain of interest relative to the non-restricting host, *L. lactis* subsp. *cremoris* MG1363.

Phage	<i>L. lactis</i> subsp. <i>cremoris</i> host	Efficiency of plaquing (EOP)
ϕ c2.MG1363	MG1363	1.0
ϕ c2.MG1363	MG1363(pCI372)	1.1
ϕ c2.MG1363	MG1363(pCI934)	0.8
ϕ c2.MG1363	UC563	1.5×10^{-5}
ϕ c2.UC563	UC563	0.8
ϕ c2.pCI934	UC563	1.2×10^{-2}

displayed considerable resistance to *ScrFI* digestion when used to infect UC563, as manifested by a 10^3 -fold increase in the EOP (Table 2). Therefore, while biochemical evidence indicated that the *ScrFI* ENase was expressed in the recombinant plasmids, pCI933 and pCI934, classical *in vivo* plaque assays suggested that it was expressed too poorly to efficiently restrict phage. Nevertheless, *in vivo* expression of the *ScrFI* MTase(s) was observed.

The role of *orfX*, if any, in the *ScrFI* R/M system is unclear. Removal of the 5' end of *orfX*, including the putative promoter region and an inverted repeat structure, was accomplished by introducing a 3.8 kb *Hae*III–*Sau*3A fragment from pCI933 into the multiple cloning site of pCI372 (Fig. 2). However, even though *E. coli* transformants containing the correct insert were successfully obtained (construct designated pCI940; Fig. 2), these chimeras appeared to be toxic to the host as it grew poorly in broth and produced only pinpoint colonies on solid media (data not shown). Subsequent retransformation of pCI940 DNA into *E. coli* gave rise to a mixed pinpoint/regular colony phenotype, with the smaller colonies correlating with the presence of the correct insert, and larger ones with a *Cm^r* deletion derivative. No further analysis was performed with these unstable constructs.

Sequence analysis and organization of the *ScrFI* R/M system

orfX, *scrFIBM* and *scrFIR* were found to be in-frame and to closely abut each other, with just 3 and 18 nucleotides between the respective ORFs, making it highly probable that all three are co-transcribed as a polycistronic message. Several sequences consistent with lactococcal promoters (van de Guchte *et al.*, 1992) were evident upstream of *orfX* (Fig. 1). Apart from an

Fig. 4. (a) Demonstration that *ScrFI* is unable to cleave pBR322 DNA at 5' CCWGG 3' sites when isolated from a *Dcm*-proficient *E. coli* host, due to the occurrence of 5mC at the 3' cytosine in these sites. The cleavage pattern generated by *ScrFI* with DNA isolated from a *Dcm*-proficient host is similar to that of *NciI*, which cleaves at 5' CCSGG 3' sites, a subset of the *ScrFI* recognition site. Lanes: 1, pBR322 (*Dcm*⁻), *EcoRII* (5' CCWGG 3'); 2, pBR322 (*Dcm*⁻), uncut; 3, DNA size markers (ϕ X174 DNA digested with *HaeIII*); 4, pBR322 (*Dcm*⁻), uncut; 5, pBR322 (*Dcm*⁻), *ScrFI* (5' CCNGG 3'); 6, pBR322 (*Dcm*⁻), *NciI* (5' CCSGG 3'); 7, pBR322 (*Dcm*⁺), uncut; 8, pBR322 (*Dcm*⁺), *ScrFI*; 9, pBR322 (*Dcm*⁺), *NciI*. (b) Assessment of the effect of non-canonical modifications in impairing *ScrFI* digestion. pBR322 DNA was isolated from a *Dcm*-proficient *E. coli* host and therefore only 5' CCSGG 3' sites are amenable to digestion by *ScrFI*. Lanes 1–3, pBR322 DNA incubated with: *ScrFI* (5 U) (lane 1); *MspI* (5 U) (lane 2); uncut (lane 3). Lanes 4–6, pBR322 DNA methylated *in vitro* with *M.SssI*: uncut (lane 4); treated with *HpaII* (5 U) (lane 5); treated with *ScrFI* (1 U) (lane 6). Lanes 7–10, pBR322 DNA methylated *in vitro* with *M.MspI*: uncut (lane 7); treated with *MspI* (5 U) (lane 8); treated with *ScrFI* (1 U) (lane 9); treated with *ScrFI* (15 U) (lane 10).

inverted repeat region which could potentially form a stem-loop structure and which was located immediately after *scrFIAM*, no other significant secondary structure

suggestive of a factor-independent transcriptional terminator was observed downstream of the other ORFs, indicating that all four genes may be transcribed as one message in an operon structure. However, the relatively larger distance of 164 nucleotides between *scrFIR* and *scrFIAM*, and the occurrence of putative promoter sequences in this intervening region, also support the possibility that *scrFIAM* may have its own independent promoter. In support of the latter proposal, a number of constructs (e.g. pCI931m) containing *scrFIAM* and its putative promoter region were capable of encoding an active MTase independent of the remainder of the *ScrFI* R/M locus (Twomey, 1996).

Comparison of *ScrFI* ENase with other proteins

The deduced amino acid sequence encoded by *scrFIR* was examined for homology with protein sequences in the databases, SWISS-PROT (release 32), GenBank (release 92) and EMBL (release 45), using the BLAST suite of programs (Altschul *et al.*, 1990). No significant homology was observed to any known proteins, including the *ScrFI* isoschizomer, *SsoII*, from *Shigella sonnei*, which also recognizes 5' [↓]CCNGG 3' but cleaves at a different location as indicated (Karyagina *et al.*, 1993). Although the sequence motif PDX_{10–30}(D/E)XK (where X is any amino acid) has been identified in a number of type II ENases, with the conserved residues forming the active site in *EcoRI* and *EcoRV* (Anderson, 1993), no such motif was identified in *ScrFI*.

Assay for activity of *ScrFI* on 5mC-modified DNA

The successful stable maintenance and expression of a type II ENase requires that recognition sites on the host genome are appropriately methylated by the cognate MTase(s) to prevent autodigestion. It has been proposed that *ScrFI* is incapable of cleaving its recognition site, 5' CCNGG 3', when the second cytosine in each strand of the duplex is 5mC, but cuts when the 5' terminal C-residue is similarly modified (McClelland *et al.*, 1994). Therefore, modification of the second C is believed to be the canonical or true modification observed in lactococcal DNA encoding the *ScrFI* R/M system (unpublished results). This was partially verified by the fact that DNA isolated from *E. coli* encoding the 5mC MTase *M.Dcm*, which modifies the internal cytosine in 5' CCWGG 3' sites (where W = A or T), remained refractory to cleavage by *ScrFI* at these sites and effectively generated an altered ENase cleavage specificity, 5' CCSGG 3' (where S = C or G; Nelson *et al.*, 1984; Fig. 4a, lanes 5 and 8). The ability of the *ScrFI* ENase to cleave non-canonically modified sites in pBR322 was investigated. As this DNA was recovered from a *Dcm*-proficient strain (*E. coli* RR1) only 5' CCSGG 3' sites are susceptible to *ScrFI* digestion. Asymmetric bimethylation of these 5' CCSGG 3' sites in which the second cytosine of one strand and the central or outer cytosine of the other strand were modified was accomplished by *in vitro* methylation with *M.SssI* (5' CG 3') and *M.MspI* (5' CCGG 3'), respectively (Figs 4b

Fig. 5. (a) Products of methylation of 5' CCSGG 3' (where S = C or G) sites modified by *M.SssI* and *M.MspI*. These modified sites may be regarded as asymmetrically modified ScrFI sites in which the second cytosine is modified on one strand and the central or outer cytosine is modified on the other strand. (b) Model to explain why double methylation of some ScrFI sequences may be required to prevent ScrFI digestion. When two ScrFI sites occur adjacent to each other, canonical modification of one of these sites (i.e. site X), prevents digestion at this site; however, since non-canonically 5mC does not completely impair ScrFI ENase digestion, the adjacent site (Y) still remains sensitive to ScrFI digestion, and needs to be further modified.

and 5a). Incubation of 1 U ScrFI ENase with either modified substrate for 1 h at 37 °C resulted in partial digestion (Fig. 4b, lanes 6 and 9), with higher enzyme concentrations (15 U; Fig. 4b, lane 10) leading to substantial restriction, illustrating that while non-canonical modifications slow down the rate of digestion, there is nevertheless an appreciable degree of digestion. Therefore, this evidence suggests that lactococcal cells expressing the ScrFI ENase need to fully protect themselves from autodigestion by canonical modification of both strands of the ScrFI recognition site.

DISCUSSION

ScrFI was the first type II ENase isolated from the industrially important lactococci and it remains the only one which is commercially available (Fitzgerald *et al.*, 1982). Identification of the ENase gene in close association with the two previously described MTase genes, *scrFIAM* and *scrFIBM* (Davis *et al.*, 1993; Twomey *et al.*, 1993), was not unexpected, as close gene linkage has been observed with all type II R/M systems characterized to date (Wilson & Murray, 1991). This close linkage presumably reflects the interdependent nature of the enzymes that constitute the system, which requires co-ordinated regulation of expression of the genes involved to permit protection of the host DNA, while still providing an effective barrier to foreign DNA.

The absence of pronounced homology between the deduced sequences of the two isoschizomers, ScrFI and SsoII, is not unusual as the primary sequences of type II

ENases are generally quite diverse and only isoschizomers which cleave at an identical position (i.e. homoschizomers) show substantial amino acid similarity (for reviews see Wilson & Murray, 1991; Roberts & Halford, 1993). In addition, the organization of the genes in these two 5' CCNNGG 3'-specific systems differs as the respective ORFs are divergently transcribed in the SsoII system, but are co-directionally transcribed in ScrFI. Therefore, despite significant amino acid identity between *M.SsoII* and the ScrFI MTases (70% and 27% identity with *M.ScrFIA* and *M.ScrFIB*, respectively), the ENases are quite different, suggesting that the cognate enzymes have independent lines of evolution or alternatively, the ENases have divergently evolved from a primitive precursor to such an extent so as to be unrecognizable by primary sequence comparisons. Furthermore, based on the ability to cleave synthetic substrates containing non-nucleotide inserts, Kubareva *et al.* (1992) have demonstrated that the ScrFI and SsoII ENases interact with their substrates in a fundamentally different manner.

Despite evidence for ENase activity in CFEs of hosts containing *scrFIR*, no apparent *in vivo* restriction of phage was observed. This result was unexpected, as the phage used in the assay contained several sites theoretically amenable to cleavage by ScrFI. Indeed, ϕ c2 has been used routinely in the laboratory to test lactococcal strains for classical ScrFI R/M activity. The reason for the absence of restriction is unclear; however, it is possible that the natural regulation between the respective ENase and MTase activities has been disrupted,

causing expression of the ENase gene to be delayed, or the increased copy number of the cloned derivatives may have altered the natural synergy between the ENase and MTase activities, allowing the phage to become fully modified prior to restriction. Alternatively, the natural expression signals for efficient restriction activity may be absent in the cloned derivatives. It is also of interest to note that phage passaged through *L. lactis* subsp. *cremoris* MG1363 harbouring pCI934 are not fully resistant to restriction in *L. lactis* subsp. *cremoris* UC563, as an EOP of less than one (1.2×10^{-2} ; Table 2) was observed. This may be due to incomplete protection of 5' CCN GG 3' sites against *ScrFI* digestion or may indicate the presence of a second R/M system in *L. lactis* subsp. *cremoris* UC563. Attempts to enhance restriction activity by cloning *scrFI*R downstream of a strong promoter (P32 in pMG36e; van de Guchte & Venema, 1989), and introducing these constructs into lactococcal hosts previously modified by the *ScrFI* MTases, were unsuccessful.

The evidence presented here suggests that *ScrFI* sites must be canonically modified on both strands to be fully resistant to *ScrFI* digestion, i.e. the second cytosines in both strands of the duplex, 5' CCN GG 3' sites must be methylated at the 5-carbon position. The rationale for studying the ability of non-canonical modifications to impair ENase cleavage in the first instance relates to the degeneracy of the *ScrFI* ENase. Since the central nucleotide in the *ScrFI* recognition site (5' CCN GG 3') is completely degenerate, in certain sequence contexts the *ScrFI* ENase may potentially cleave its target sequence, even if some non-canonical bases within this site have already been modified by one of the *ScrFI* MTases. An example of such a sequence is 5' CCC GGG 3' (i.e. a *SmaI* site) which is essentially two adjacent overlapping *ScrFI* sites (Fig. 5b, X and Y). If one of the *ScrFI* sites (X) is canonically modified on both strands by either of the *ScrFI* MTases, it should be refractory to cleavage. However, the adjacent site (Y) is non-canonically modified and is potentially susceptible to cleavage, requiring further modification to be fully protected. Therefore, some *ScrFI* sites, which would be predicted to occur rarely on the lactococcal genome, may need to have two adjacent cytosines methylated on the one strand to prevent the degenerate ENase from cleaving. Considering that many MTases are impaired by non-canonical modifications within their target sequence (Nelson & McClelland, 1987), modification of two adjacent cytosines may require two fundamentally different MTases, which differ with respect to their ability to be sterically hindered by prior modifications in their recognition site. The *ScrFI* R/M system is unique among characterized R/M systems in that it is the only one to date known to have two 5mC MTases associated with an ENase which recognizes a symmetrical recognition sequence. The function of these two MTases remains to be established but the ability of *ScrFI* to cleave non-canonically methylated sites suggests that the two *ScrFI* MTases may have evolved to counteract the degeneracy of the *ScrFI* ENase.

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