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

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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-
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; Pösfai 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 similar significances 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 (Terrarighi & Sandine, 1975) containing either glucose or lactose as required 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 &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 preheated 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 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 disruptor (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 &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 preheated 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 (RK1) and 1 µg was methylated in vitro with 6 U of M.SssI or M.MspI in the presence of 80 µM 5-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

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Relevant genotype or phenotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>Δ(mcrA)183 Δ(mcrBC–hsdSMR–mrr)173 endA1 supE44 thi-1 recA1</td>
<td>Stratagene</td>
</tr>
<tr>
<td></td>
<td>XL1-Blue MRF</td>
<td></td>
</tr>
<tr>
<td>RR1</td>
<td>F ’Δ(gpt–proA)62 leu supE44 ara-14 galK2 lacY1 Δ(mcrC–mrr)</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>rpsL20(Str+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>xyl-5 met-1 recA1</td>
<td></td>
</tr>
<tr>
<td>L. lactis subsp. cremoris</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1363</td>
<td>Plasmid-free derivative of L. lactis subsp. cremoris 712</td>
<td>Gasson (1983)</td>
</tr>
<tr>
<td>UC503</td>
<td>Isolate from Irish Cheddar cheese mixed-strain starter encoding ScrFI</td>
<td>Fitzgerald et al. (1982)</td>
</tr>
<tr>
<td></td>
<td>R/M activity</td>
<td>Costello (1988)</td>
</tr>
<tr>
<td>UC563</td>
<td>Derivative of L. lactis subsp. cremoris UC503 cured of the phage-resistance adsorption-blocking plasmid pCIS28</td>
<td></td>
</tr>
</tbody>
</table>

| Bacteriophage | | |
|              | | |
| pB2          | Prolate-headed lactococcal phage susceptible to ScrFI restriction | UCC Phage Collection |
| Plasmids     | | |
| pCI372       | 5.7 kb E. coli–L. lactis shuttle vector, Cm’ | Hayes et al. (1990) |
| pCI931m      | pBR322-based chimeric construct containing scrFIM modification gene from L. lactis subsp. cremoris UC503, Ap’ | Davis et al. (1993) |
| pCI932m      | pBR322-based chimeric construct containing scrFBM modification gene from L. lactis subsp. cremoris UC503, Ap’ | Davis et al. (1993); Twomey (1996) |
| pCI933       | pBluescript-based construct with a 4.2 kb NruI-Sau3A fragment from L. lactis subsp. cremoris UC503 containing scrFZBM, scrFIR and scrFAM, Ap’ | This study |
| pCI934       | pCI372-based construct with 4.2 kb XhoI–Sau3A fragment from pCI933 containing orfX, scrFBM, scrFIR and scrFAM, Cm’ | This study |
| pCI940       | pCI1372-based construct with a 3.8 kb HaeIII–Sau3A fragment from pCI933 containing scrFBM, scrFIR and scrFAM, Cm’ | This study |
| pCI942       | Derivative of pCI933 with an EcoRI–Ndel deletion internal to scrFIR, Ap’ | This study |
| pBluescript KS I | E. coli cloning vector, Ap’ | Stratagene |
| pBR322       | E. coli cloning vector, Ap’ Tet’ | Bolivar et al. (1977) |

The amount of enzyme required to fully protect 1 μg of DNA in 1 h at 37 °C in a 10 μl 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 μg of 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 SmC 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 SmC MTase genes from pCI931m and pCI932m have been previously reported (Davis et al., 1993; Twomey et al., 1993). The sequences of the
Fig. 1. Completed nucleotide sequence of the ScrF R/M system and deduced amino acid sequences encoded by orfX, scrFBIM, scrFI R, and scrFIAM. The sequences of two 5mC MTase genes, scrFlAM, and scrFIBM have been reported previously. The direction of transcription is illustrated by short arrows following the gene designations. Sequences equivalent to promoter sequences are boxed. Predicted ribosome-binding sites are denoted RBS and underlined. Facing arrows indicate no. L12227) and (Davis 1993; GenBank accession no. M87289) have been reported previously. The direction...
The 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 <i>L. lactis</i> 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 pBlue-script KS I, followed by the introduction of a 2.0 kb NruI–EcoRI fragment from pCI932m (Fig. 2). This construct, designated pCI933, was electrotansformed into <i>E. coli</i> 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, <i>L. lactis</i> subsp. cremoris UC503, and which was absent in the plasmid-free <i>E. coli</i> 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 <i>L. lactis</i>, the shuttle vector pCI372, which contains the replication regions of plasmids pCI305 (isolated from <i>L. lactis</i> subsp. lactis UC317) and pBR322, was employed to reintroduce the fragment of interest into <i>L. lactis</i>. An XhoI-Sau3A fragment from pCI933 was subcloned into the compatible SalIIBamHI sites within the multiple cloning site of pCI372 (generating pCI934), and transformed into plasmid-free <i>L. lactis</i> subsp. cremoris MG1363. Analysis of cell-free extracts from transformants harbouring the
pCI934 construct exhibited ENase activity, although the activity was poor relative to \textit{L. lactis} subsp. \textit{cremoris} UC503 and \textit{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 this, evidence indicated that the ScrFI ENase was expressed too poorly to efficiently restrict phage.

When \textit{L. lactis} subsp. \textit{cremoris} MG1363 harbouring pCI934 was examined for \textit{in vivo} restriction of the prolate-headed lactococcal phage \textphi{2}, no significant restriction was observed (Table 2). In contrast, substantial restriction was observed with \textit{L. lactis} subsp. \textit{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 \textit{et al.}, 1992; Davis \textit{et al.}, 1993). Evidence that the ScrFI R/M system contributed to the 10-fold reduction in EOP observed, rather than an alternative R/M system encoded by UC563 being wholly responsible, was provided by purifying \textphi{2} which had been passaged through MG1363 (pCI934) (containing both \textit{scrFIA} and \textit{scrFIBM}) and reinfecting the ScrFI-proficient host, UC563. Phage recovered from MG1363 (pCI934) were methylated by the ScrFI MTase(s) and displayed considerable resistance to ScrFI digestion when used to infect UC563, as manifested by a 10\textsuperscript{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 \textit{in vivo} plaque assays suggested that it was expressed too poorly to efficiently restrict phage. Nevertheless, \textit{in vivo} expression of the ScrFI MTase(s) was observed.

The role of \textit{orfX}, if any, in the ScrFI R/M system is unclear. Removal of the 5' end of \textit{orfX}, including the putative promoter region and an inverted repeat structure, was accomplished by introducing a 3.8 kb \textit{HaeIII–Sau3A} fragment from pCI933 into the multiple cloning site of pCI372 (Fig. 2). However, even though \textit{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 \textit{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 Cmr deletion derivative. No further analysis was performed with these unstable constructs.

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

<table>
<thead>
<tr>
<th>Phage</th>
<th>L. lactis subsp. cremoris host</th>
<th>Efficiency of plaquing (EOP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textphi{2}.MG1363</td>
<td>MG1363</td>
<td>1.0</td>
</tr>
<tr>
<td>\textphi{2}.MG1363</td>
<td>MG1363(pCI372)</td>
<td>1.1</td>
</tr>
<tr>
<td>\textphi{2}.MG1363</td>
<td>MG1363(pCI934)</td>
<td>0.8</td>
</tr>
<tr>
<td>\textphi{2}.MG1363</td>
<td>UC563</td>
<td>1.5 \times 10\textsuperscript{-5}</td>
</tr>
<tr>
<td>\textphi{2}.UC563</td>
<td>UC563</td>
<td>0.8</td>
</tr>
<tr>
<td>\textphi{2}.pCI934</td>
<td>UC563</td>
<td>1.2 \times 10\textsuperscript{-2}</td>
</tr>
</tbody>
</table>

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

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

\textphi{2}.MG1363, \textphi{2}.UC563 and \textphi{2}.pCI934 refer to \textphi{2} recovered from single-plaque isolates following infection of \textit{L. lactis} subsp. \textit{cremoris} MG1363, UC563 and MG1363(pCI934), respectively. EOP data are expressed as the ability of \textphi{2} to infect the strain of interest relative to the non-restricting host, \textit{L. lactis} subsp. \textit{cremoris} MG1363.

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

\textit{orfX}, \textit{scrFIBM} and \textit{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 \textit{et al.}, 1992) were evident upstream of \textit{orfX} (Fig. 1). Apart from an
which cleaves at 5' CCSGG 3' sites, a subset of the fig. 4. (a) Demonstration that (lane 2); uncut (lane 3). Lanes 4-6, pBR322 DNA methylated in vitro with Dcm', 3'); 7, pBR322 (Dcm'), uncut; 8, pBR322 (Dcm'), treated with Mspl. The cleavage pattern generated by ScrFI with DNA isolated from a Dcm-proficient host is similar to that of NciI, which cleaves at 5' CCWG 3' sites, a subset of the ScrFI recognition site. Lanes: 1, pBR322 (Dcm'), EcoRll (5' CCWGG 3'); 2, pBR322 (Dcm'), uncut; 3, DNA size markers (pX174 DNA digested with Haelll); 4, pBR322 (Dcm'), uncut; 5, pBR322 (Dcm'), ScrFI (5' CCNNGG 3'); 6, pBR322 (Dcm'), NciI (5' CCSSGG 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 Hpal (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).

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. pCD31m) 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, SsOl, from Shigella somei, which also recognizes 5' ICCNGG 3' but cleaves at a different location as indicated (Karyagina et al., 1993). Although the sequence motif PDXp10(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.

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' CCSSGG 3' sites, a subset of the ScrFI recognition site. Lanes: 1, pBR322 (Dcm'), EcoRll (5' CCWGG 3'); 2, pBR322 (Dcm'), uncut; 3, DNA size markers (pX174 DNA digested with Haelll); 4, pBR322 (Dcm'), uncut; 5, pBR322 (Dcm'), ScrFI (5' CCNNGG 3'); 6, pBR322 (Dcm'), NciI (5' CCSSGG 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 Hpal (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

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' CCWG 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' CCSSGG 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' CGG 3'), respectively (Figs 4b

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Sequence of the ScrFI R/M system
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'CNGG 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.ScrFI A 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, φC2 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 passed through \textit{L. lactis} subsp. \textit{cremoris} MG1363 harbouring pCI934 are not fully resistant to restriction in \textit{L. lactis} subsp. \textit{cremoris} UC563, as an EOP of less than one (1.2 x 10^{-3}; Table 2) was observed. This may be due to incomplete protection of 5' CCNGG 3' sites against \textit{ScrFI} digestion or may indicate the presence of a second R/M system in \textit{L. lactis} subsp. \textit{cremoris} UC563. Attempts to enhance restriction activity by cloning \textit{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 \textit{ScrFI} MTases, were unsuccessful.

The evidence presented here suggests that \textit{ScrFI} sites must be canonically modified on both strands to be fully resistant to \textit{ScrFI} digestion, i.e. the second cytosines in both strands of the duplex, 5' CCNGG 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 \textit{ScrFI} ENase. Since the central nucleotide in the \textit{ScrFI} recognition site (5' CCNGG 3') is completely degenerate, in certain sequence contexts the \textit{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 \textit{ScrFI} MTases. An example of such a sequence is 5' CCCCGG 3' (i.e. a \textit{Smal} site) which is essentially two adjacent overlapping \textit{ScrFI} sites (Fig. 5b, X and Y). If one of the \textit{ScrFI} sites (X) is canonically modified on both strands by either of the \textit{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 \textit{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 \textit{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 \textit{ScrFI} to cleave non-canonically methylated sites suggests that the two \textit{ScrFI} MTases may have evolved to counteract the degeneracy of the \textit{ScrFI} ENase.

ACKNOWLEDGEMENTS

This work was supported by grants from the European Community BRIDGE (contract BIOT-CT91-0263[SSMA]) and BIOTECH (contract B02-CT94-3055) programmes and by the Food Sub-Programmes of the Operational Programme for Industrial Development, which is administered by the Irish Department of Agriculture Food and Forestry and supported by national and European Union funds.

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Received 4 December 1996; revised 3 March 1997; accepted 7 March 1997.