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Genetic analysis of a lactococcal plasmid replicon

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Summary. The sequence and genetic organization was determined of the 2508 bp lactococcal portion of pFX2, which was derived from a cryptic *Lactococcus lactis* subsp. *lactis* plasmid and used as the basis for construction of a series of lactococcal vectors. A lactococcal plasmid plus origin and two replication protein-coding regions (*repA* and *repB*) were located. RepA has a helix-turn-helix motif, a geometry typical of DNA-binding proteins. RepB shows a high degree of homology to the plasmid replication initiation proteins from other gram-positive bacteria and *Mycoplasma*. The transcribed inverted repeat sequence between *repA* and *repB* could form an attenuator to regulate pFX2 replication. Upstream of the *ori* site, and in a region which was non-essential for replication, a 215 bp sequence identical to the staphylococcal plasmid pE194 and carrying the RS_A site was identified. The genetic organization of this lactococcal plasmid replicon shares significant similarity with pE194 group plasmids.

Key words: Lactococcal plasmid pFX2 – DNA sequence – Genetic organization – Replicon – Replication regulation

Introduction

Cloning vectors for gram-positive organisms were initially constructed as chimeric plasmids carrying replicons from both gram-positive and -negative organisms to enable replication in these two genetically divergent groups of bacteria. Recently, however, a new class of lactococcal vectors has been constructed based solely on the replicons from small cryptic lactococcal plasmids: pWV01 (2.3 kb) from *Lactococcus lactis* subsp. *cremoris* Wg2 (Kok et al. 1984), pSH71 (2.1 kb) from *L. lactis* subsp. *lactis* 712 (de Vos 1987) and pD125 (5.5 kb) from *L. lactis* subsp. *lactis* 5136 (Xu et al. 1990, 1991). These lactococcal vectors are functional in both lactococci and *Escherichia coli* and have been used extensively for gene cloning studies (de Vos 1987; Xu et al. 1990, 1991).

In our laboratory, the lactococcal vector pFX1 was constructed by combining the 4.5 kb *Hpa*II-*Mbo*I fragment of pDI25 with a 1 kb fragment carrying the chloramphenicol acetyl transferase (*cat*) structural gene from staphylococcal plasmid pC194. Plasmid pFX2 was derived from pFX1 by deletion of a non-essential 1.9 kb *Cla*I region (Xu et al. 1990). Based on pFX2, a family of lactococcal vectors was constructed for gene cloning and translational fusion studies (Xu et al. 1991).

The genetic organization of many plasmid replicons from gram-positive bacteria such as *Staphylococcus* (Novick 1989), *Bacillus* (Gruss and Ehrlich 1989), *Streptococcus* (del Solar et al. 1989), *Lactobacillus* (Bates and Gilbert 1989), *Streptomyces* (Kendall and Cohen 1988) and *Clostridium* (Garnier and Cole 1988) is now well established. In contrast, very little is known about lactococcal plasmid replicons at the molecular level. A plus origin (*ori*) site has been found in lactococcal plasmid pSH71 (Gruss and Ehrlich 1989) and the minimum replicon regions located in two other plasmids from *L. lactis* subsp. *lactis* strains UC317 (Hayes et al. 1990) and SSD207 (von Wright et al. 1990). In lactococci, several industrially important genes are plasmid encoded, such as those for lactose utilization, proteinase and citrate utilization. The elucidation of plasmid replication mechanisms is fundamental to further vector development and to understanding gene expression in lactococci. In this paper, we describe the sequence and genetic organization of pFX2 DNA.

Materials and methods

pFX2 sequence determination. pFX2 DNA was linearized with *Hpa*II and cloned into *Sma*I sites of the sequencing vectors pGEM3Z and pGEM4Z (Promega). Overlapping templates were created by exonuclease III digestion with the Erase-a-Base™ system (Promega). The DNA

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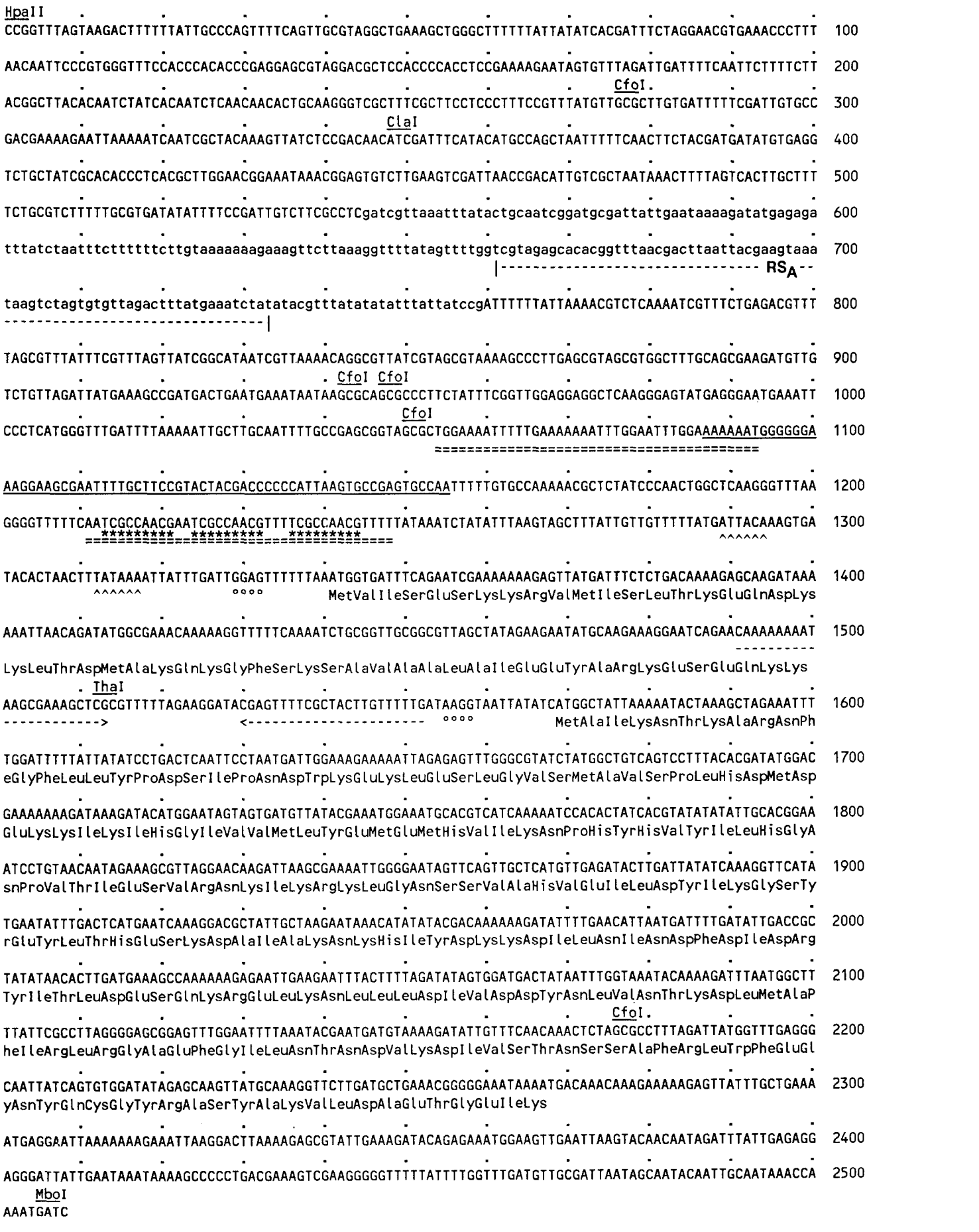
HpaII
CfoI
ClaI
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CfoI CfoI
CfoI
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ThaI
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GluLysLysIleLysIleHisGlyIleValValMetLeuTyrGluMetGluMetHisValIleLysAsnProHisTyrHisValTyrIleLeuHisGlyA
snProValThrIleGluSerValArgAsnLysIleLysArgLysLeuGlyAsnSerSerValAlaHisValGluIleLeuAspTyrIleLysGlySerTy
rGluTyrLeuThrHisGluSerLysAspAlaIleAlaLysAsnLysHisIleTyrAspLysLysAspIleLeuAsnIleAsnAspPheAspIleAspArg
TyrIleThrLeuAspGluSerGlnLysArgGluLeuLysAsnLeuLeuLeuAspIleValAspAspTyrAsnLeuValAsnThrLysAspLeuMetAlaP
CfoI
heIleArgLeuArgGlyAlaGluPheGlyIleLeuAsnThrAsnAspValLysAspIleValSerThrAsnSerSerAlaPheArgLeuTrpPheGluGl
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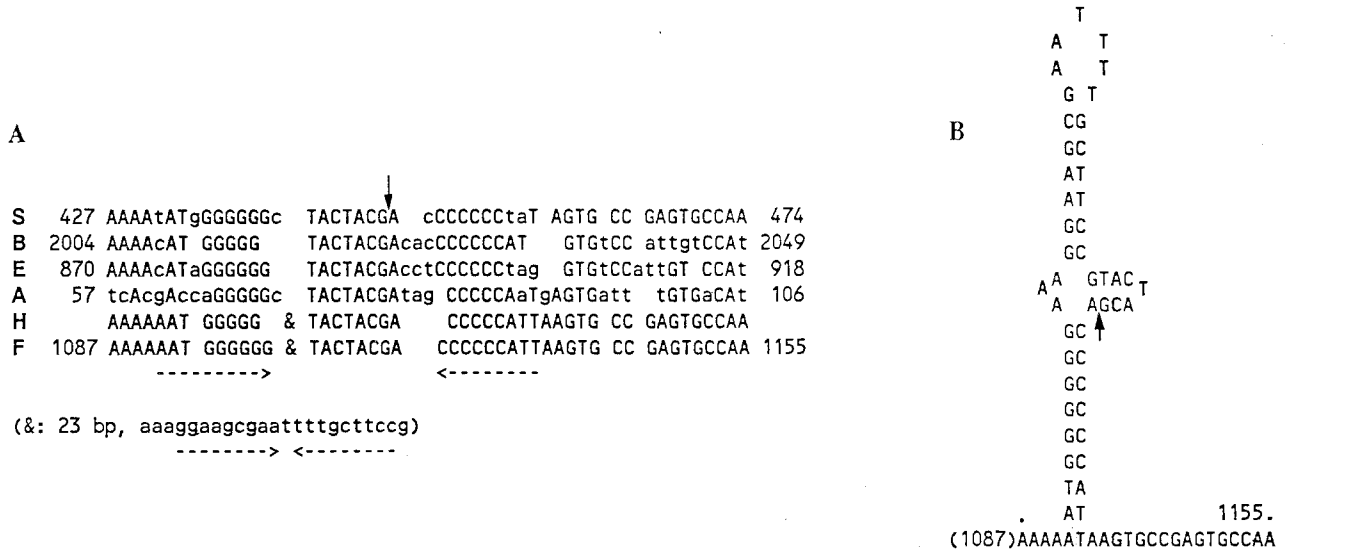


Fig. 2A and B. The plus *ori* site of lactococcal plasmid pFX2. **A** Comparison of plasmid plus *ori* sites. The gaps in the sequence alignment have been introduced for maximum homology. S, pLS1; B, pLB4; E, pE194; A, pADB201; H, pSH71; F, pFX2. Inverted repeat sequences are indicated by *facing arrows* and the nicking site in pLS1 is *arrowed*. The conserved, inverted sequence of 23 bp of pFX2 and pSH71 is shown below. **B** A loop structure in the plus *ori* locus of lactococcal plasmid pFX2 ($\Delta G° = -22$ kcal/mol). The possible nicking site is *arrowed*

sequence of each strand was determined using sequenase (USB) and the dideoxynucleotide chain-termination method (Sanger et al. 1977) in the presence of [α-^{35}S] dATP (Amersham). All enzymes were used according to the recommendations of the manufacturers. The software package developed by the University of Wisconsin Genetics Computer Group, USA was used for analyses of DNA sequences and translated peptides.

DNA manipulation. L. lactis subsp. *lactis* 4125 (Crow et al. 1983) and *E. coli* JM109 (Yanisch-Perron et al. 1983) were transformed by electroporation as previously described (Xu et al. 1990). Other methods of DNA manipulation were according to Maniatis et al. (1982).

Results and discussion

General features of the sequence

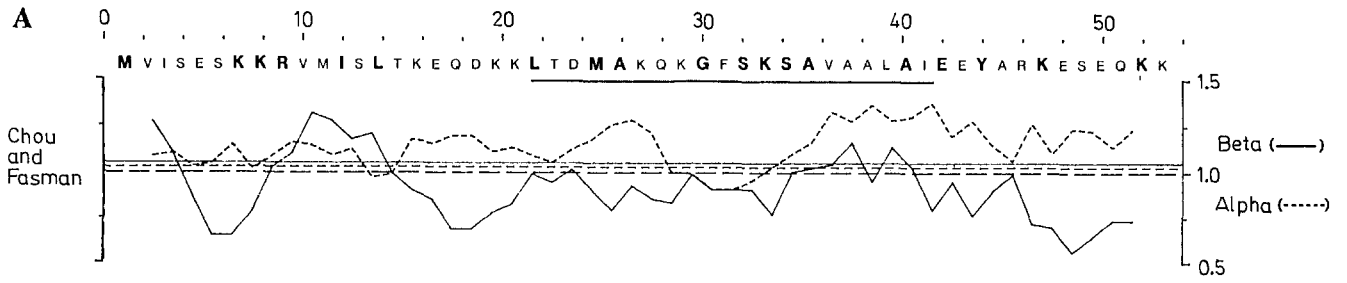
As the *cat* gene DNA sequence has been described previously (Horinouchi and Weisblum 1982), only the lactococcal portion of pFX2 (2508 bp) is presented here (Fig. 1). The G+C content of the lactococcal DNA was 35.2%, within the range for lactococci (34.4%–36.3%) reported by Garvie et al. (1981). The complete pFX2 DNA sequence including the *cat* gene sequence (1032 bp) was 3536 bp, close to the size of 3.6 kb estimated by agarose gel analysis (Xu et al. 1991). The establishment of the pFX2 sequence enables pFX3 (Xu et al. 1991) to be used as a sequencing vector, as the entire pFX3 sequence (4473 bp) comprises the known sequences of pFX2, *lacZ'* and the polylinker regions. All polylinker restriction sites in pFX3 and in pFX4, 5 and 6 (Xu et al. 1991) are unique and hence available for cloning.

Fig. 1. Nucleotide sequence and inferred amino acid sequences of the lactococcal portion (*Hpa*II-*Mbo*I) of pFX2. Coding regions are RepA (positions 1341–1499) and RepB (positions 1569–2267). Nucleotides in *lower case* letters represent the region identical to staphylococcal plasmid pE194. A possible plus *ori* site (*solid underline*) and direct repeat regions (*double dashed line*) are indicated. Within the second direct repeat region are three iterons (indicated by *asterisks*). The inverted repeat region that could form an attenuator is indicated by *facing arrows*. Putative promoter regions and ribosome binding sites are indicated below the sequence by *inverted letter* v and *open circles* respectively. RS_A and important restriction sites are marked

Lactococcal plasmid plus ori *site*

The essential region for plasmid pFX2 replication has been previously located within a 1.2 kb *Cfo*I-*Tha*I-*Cfo*I region (Xu et al. 1991), i.e. positions 1050–2176. By comparing this sequence with three classes of plus origin sequences (Gruss and Ehrlich 1989), a region homologous to the plus *ori* sites of pE194 group plasmids was identified (Fig. 2). Recently, the precise nicking site in pLS1 was located between bases G (448) and A (449) in the *ori* site (Fig. 2A) (de la Campa et al. 1990). The pFX2 *ori* sequence differed slightly from that of lactococcal plasmid pSH71 with additional bases G (1099) and C (1131). The location of the *ori* site in pFX2 was confirmed by deleting the region between positions 1 and 1151 with exonuclease III, making the plasmid nonfunctional in lactococci.

In contrast with the *ori* sequences of other plasmids in this group, lactococcal plasmids pFX2 and pSH71 have an additional conserved, inverted sequence of 23 bp, forming an extended stem-loop structure with a



B

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F  MAI------------KNTKA-------RNFGFLLYPDSIPNDWKEKLESLGVSMAVSPLHDMDEK----KIKIHGIVVMLYEMEMHVIKNPHYHVYILHG
S  MA--------------KEKA-------RYFTFLLYPESIPSDWELKLETLGVPMAISPLHDKDKS----SIK------------GQKYKKAHYHVLYIAK
B  MKSESKIDWTVPRPNKNPKTKQPYKRGRNWGIVVYPESLPENW--KDIIRQEPIAVSPLHDKDVNPDGEKKKSHYHLVLNYKGNKSFEQIDEIARSLRAP
E  MSENVIKETENK---KNSRG-------RNWTFVLYPESAKAEWLEYLKELHIQFVVSPLHDRD------------------TDTEGRMKKEHYH--ILVM
A  M-S------------TNIKK-------RNWTLLVYPDSAPENWKEILDQNGV-EYFGALHDKD------------------VNPDGTIKKPHYHIVLAYS

F  NPVTIESVRNKIKRKLGNSSVAHVEILDYIKGSYEYLTHESKDAIAKNKHIYDKKDILNINDFDIDRYITLDESQKRELKNLLLDIVDDYNLVNTKDLM-
S  NPVTADSVRKKIKLLLGEKSLAMVQVVLNVENMYLYLTHESKDAIAKKKHVYDKADIKLINNFDIDRYVTLDVEEKTELFNVVVSLIRAYTLQNIFDLY-
B  APQRISS----------------------LTGAVRYLTH----MDNPEKYQYDNADIETFGGFDLESCLALSTGDKRQALRDMLAFISENEIMHLKDFA-
E  YEGNKSYEQIKIITEELN------ATIPQIAGSVKGLVRYMLHMDDPNKFKYQKEDMIVYGGVDVDELLKKTTTDRYKLIKEMIEFIDEQGIVEFKSLMD
A  GPTT----FNNVKTLCNTLNSPKPLPLDGVGGMWRYMTH----KDNPEKYQYDDSIIFTGNGFDISNYKELTKKEISDIKLGLIDI-------IKNKQI-

F  -AFIRLRGAEFGILNTNDVKDIVSTNSSAFRLWFEGNYQCGYRASYAKVLDAETGEI----------------------K          (233)
S  -DFIDENG-ETYGLTINLVNEVIAGKTGFMKLLFDGAYQRSKR--------GTKNEE----------------------R          (210)
B  -DYCMSEEAPAG---------------WFELLTERN---------------TLFIKEYIKSNWQKQQQYASKNINKMSD           (217)
E  YAMKFKFDDWFPLLCDNSAYVI----------------QEYIKSNRYKSD----------------------------R          (199)
A  -TEYSTFIDVVSNLGNIDVFDVASKNTIFF---------TSYINSFRFKLREEMEQEKYT-------------------K          (196)
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Fig. 3A and B. Replication proteins of lactococcal plasmid pFX2. **A** Graph (PEPPLOT) of α-helix and β-sheet probabilities in the RepA secondary structure, predicted according to Chou and Fasman (1974). *Bold type* letters represent the amino acids homologous to RepA of pLS1, and the 20-residue helix-turn-helix motif showing consensus to the major domain of DNA-binding proteins is *underlined*. **B** Comparison of RepB(pFX2) with plasmid replication initiation proteins. F, pFX2(RepB); S, pLS1(RepB); B, pLB4(RepB); E, pE194(RepF); A, pADB201(RepA). The total number of residue of these peptides are indicated in *brackets*

possible nicking site on the side of the stem (Fig. 2B). A stem-loop structure in the pLS1 *ori* site has been found to be the signal for plasmid replication initiation (de la Campa et al. 1990). Two regions carrying direct repeats were found flanking the *ori* site, at positions 1054–1093 and 1211–1148. The latter direct repeat region was similar to the direct repeat region (iterons) of pLS1 (positions 534–566) (del Solar et al. 1989) with respect to GC-content (50% G+C), number of repeats (3 repeats; 11 bp for pLS1 and 9 bp for pFX2) and in the repeat position relative to the *ori* and promoter regions for the replication proteins. In pLS1, the region with the three direct repeats was shown to be involved in the initiation protein and repressor binding (Puyet et al. 1988; del Solar et al. 1989; de la Campa et al. 1990).

Lactococcal plasmid replication proteins

Downstream of the *ori* site, two open reading frames ORF1 and ORF2 were located, with 54 and 234 codons respectively (Fig. 1). Eight bp upstream from the ATG site of ORF1 (positions 1341–1343), there was a potential ribosome-binding site (RBS) GGAG ($\Delta G° = -9.4$ kcal/mol), preceded 11 bp upstream by a lactococcal promoter sequence TATAAA (−10) and ATTACA (−35) separated by 17 bp. This promoter arrangement is

homologous to those published previously (Lakshmidevi et al. 1990; van der Vossen et al. 1987). The region from positions 1241 to 1328 including the promoter and upstream DNA was very AT-rich (82%), similar to the strong promoter regions in *Bacillus subtilis* (Doi 1984). The −10 and −35 hexamers were also similar to the *E. coli* consensus promoter (TATAAT and TTGACA, respectively) (Harley and Reynolds 1987), which probably explains why pFX2 is functional in *E. coli*. Ten bp upstream from the ATG start codon of ORF2 (positions 1569–1571), another possible RBS was located, AAGG ($\Delta G° = -8.4$ kcal/mol).

ORF1 (*repA*) encoded a predicted 53-residue peptide with molecular mass of 6.0 kDa. The RepA amino acid sequence had a high degree of homology with the translated peptides which precede the replication initiation proteins from pLS1 (RepA), pADB201 (RepB), pLB4 (RepA) and pE194 (RepG) (Bates and Gilbert 1989). Prediction of the secondary structure and characteristics of the RepA peptide of pFX2 was determined using protein analysis programs. RepA had a predicted isoelectric point of 10.50, and was positively charged due to its 12 strongly basic residues. The secondary structure of RepA predicted by the PEPPLOT program showed a helix-turn-helix motif, typical of many DNA-binding proteins (Pabo and Sauer 1984) (Fig. 3). This motif extended from residues 16–27 (helix-1), and from residues 34–52 (helix-2). A β-sheet structure was observed between residues 10 and 13. A flexible segment was also found between residues 28–33 (the turn motif). The central helix-turn-helix 20-residue (22–41) motif of RepA fitted well with the requirements proposed for binding site geometries of repressor proteins (Pabo and Sauer 1984; Ohlendorf et al. 1983). Conserved hydrophobic A, G and V residues were located at the relative positions 26, 30 and 36. Residues at the relative positions 25(M), 31(F), 39(L), 40(A) were also hydrophobic. Hydrophilic residues were at positions 24(D) and 28(Q). RepA of pFX2 showed strong homology with the RepA repressor of pLS1, especially in the N-terminal and helix-turn-helix domains (Fig. 3). The relatively conserved N-terminal domains in repressors (Lambda repressors, RepA(pLS1) and TrfB (RK2) are believed to play a functional role in DNA binding (Jordan and Pabo 1988; del Solar et al. 1989).

ORF2 (*repB*) encoded a 233-residue peptide with molecular mass of 26.9 kDa with no homologous promoter region, suggesting polycistronic transcription with ORF1. The well-characterized replication initiation proteins of pT181 (Novick et al. 1989) and pLS1 (Puyet et al. 1988) also do not have their own separate promoters. The RepB amino acid sequence showed substantial homology throughout the whole sequence to the pLS1 replication initiation protein (RepB) (de la Campa et al. 1990), but homology only to the N-terminal regions of replication initiation proteins from pE194(RepF) (Villafane et al. 1987), pLB4(RepB) (Bates and Gilbert 1990) and pADB201(RepA) (Bergemann et al. 1989) (Fig. 3). RepB of pLS1 has recently been shown to bind at the three-direct-repeat region, and plasmid replication is initiated at the nicking site 86 bp upstream from the first basepair of the direct repeats (de la Campa et al. 1990). In pFX2, the putative nicking site is 84 bp upstream from the three iterons. RepB is therefore most likely to be the replication initiation protein of pFX2.

It was previously shown that the 1.2 kb *Cfo*I-*Tha*I-*Cfo*I region (positions 1050–2176) could be ligated to give a functional replicon (Xu et al. 1991). In this construct, 29 amino acids had been deleted from the C-terminus without affecting replication function. A similar observation was reported with pE194 where 43 C-terminal amino acids of the replication protein were found to be non-essential (Villafane et al. 1987). A cointegrate plasmid of pFX2/*Tha*I and *E. coli* plasmid pUBS/*Ssp*I was previously shown to replicate in *E. coli* but not in lactococci (Xu et al. 1991), suggesting that the insertion in the *Tha*I site of pFX2 disrupted RNA transcription through *repB*. The failure of this cointegrate plasmid to replicate in lactococci was unlikely to be due to the *E. coli* insert, because another similar construct (pFX2/*Hpa*II and pGEM3Z/*Sma*I) expressed the Cm^r marker in lactococci.

Most plasmids so far examined from gram-positive bacteria replicate by a rolling circle mechanism via a single-stranded DNA (ssDNA) intermediate (Gruss and Ehrlich 1989). Plasmids showing DNA or amino acid sequence homology to pFX2 are of the ssDNA replication type, suggesting that pFX2 also replicates in this manner. It has been demonstrated that insertion of pBR-type DNA into plasmids of ssDNA type results in the generation of high-molecular-weight plasmid multimers (Gruss and Ehrlich 1989); such forms could also be seen with the cointegrate plasmids of pFX2 and pGEM3Z (data not shown). By analogy with pE194 (Scheer-Abramowitz et al. 1981) and pLS1 (Puyet et al. 1988), pFX2 replication would then proceed towards the *Mbo*I site, i.e. anticlockwise on the circular map (Xu et al. 1991).

Possible regulatory mechanisms for lactococcal plasmid replication

Regulation systems for plasmid replication have been extensively studied for plasmids ColE1 (Tomizawa 1986) and IncFII (Praszkier et al. 1989) from gram-negative bacteria and plasmids pT181 (Novick 1989), pLS1 (del Solar et al. 1989), pC194 (Alonso and Tailor 1987) and pE194 (Villafane et al. 1987) from gram-positive bacteria. These systems all involved negative regulation of the synthesis of replication initiation proteins by antisense RNA. In pLS1, the repressor protein (RepA) was shown to bind specifically to the operator/promoter region of the *repAB* polycistron (del Solar et al. 1989). Considering the similar structure and organization in replication proteins and their promoter regions, RepA of pFX2 might also bind to a putative operator/promoter region and prevent RNA polymerase from binding. This would be a self-regulated feedback mechanism as the *repA* and *repB* of pFX2 appear to be transcribed from the same operon.

Recently, plasmid replication regulation by transcription attenuation was proposed for plasmid pT181 (Novick et al. 1989). A similar attenuation model is postulated here as a second regulatory mechanism for pFX2 replication. In the presence of the appropriate regulators, a rho-independent terminator structure consisting of a GC-rich hairpin followed by a stretch of U-residues

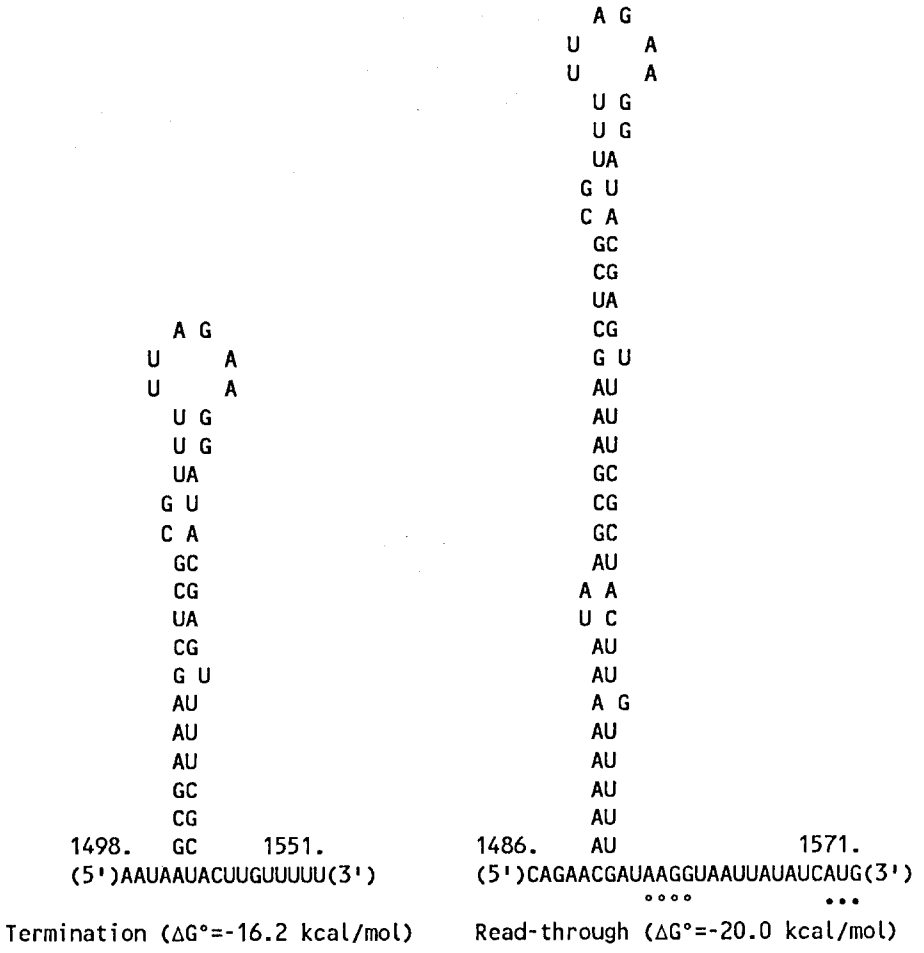

Fig. 4. Computer-generated transcriptional attenuation model for regulation of lactococcal plasmid replication. Bases marked with *open circles* represent the potential ribosome-binding site; *dots* indicate the start codon for RepB(pFX2)

(Rosenberg and Court 1979) could be formed in the inverted repeat region between *repA* and *repB* (Fig. 4). Terminators of this type have been found in several lactococcal genes (de Vos 1987) but not in the replicons of other pE194 group plasmids. The terminator would prevent RNA polymerase from transcribing through to the *repB*. In the absence of the regulators, a RNA polymerase read-through structure could be formed (Fig. 4) in the same inverted repeat region. The regulators in this system might be antisense RNA as was found with pT181 plasmid (Novick et al. 1989), and here could bind to a region upstream from the inverted repeat sequence, preventing pairing of the A-rich (positions 1491–1497) and U-rich (positions 1546–1552) regions.

A region identical to staphylococcal plasmid pE194

The pFX2 sequence contains a 215 bp region (positions 545–759) with 100% homology to staphylococcal plasmid pE194 (positions 2925–3139) (Horinouchi and Weisblum 1982). In pE194, this region carried the complete RS_A (recombination site A) locus and the staphylococcal *pre* (plasmid recombination enzyme) promoter region (Gennaro et al. 1987) (Fig. 1). In pFX2, however, there was no analogous ORF for the staphylococcal consensus Pre protein which nicks specifically at RS_A (Gennaro et al. 1987). Staphylococcal plasmid pE12 has also been reported to carry RS_A without the *pre* (Novick 1989). Plasmid recombination/cointegration mediated by the pre-RS_A process is common in staphylococci and bacilli (Gruss and Ehrlich 1989; Novick 1989) and is *recA* independent (Gennaro et al. 1987). RS_A has also been postulated to act as the *oriT* site for conjugative mobilization of streptococcal plasmid pMV158 (Priebe and Lacks 1989).

In *E. coli*, small plasmids such as ColE1, which cannot mediate their own transfer during conjugation, can be mobilized by a conjugative plasmid. A mobilization protein (Mob), which is encoded by this small plasmid, nicks at a specific site *oriT* on the plasmid. Palindromic sequences are found near the *oriT* sites of six plasmids from gram-negative bacteria (two conjugative, F and RK2; and four non-conjugative, ColE1, CloDF13, pSC101 and RSF1010) (Willetts and Wilkins 1984). Seven plasmids from different gram-positive bacteria (pUB110, pMV158, pTB913, pT181, pE194, pNE131 and pT48) are also known to carry almost identical palindromic sequences in the putative *oriT* site RS_A (Priebe and Lacks 1989; van der Lelie et al. 1989). The RS_A site in pFX2 might also act as a generalized nicking site for the formation of cointegrate plasmids during conjugative mobilization. While no Pre-like proteins to mediate this process are encoded downstream of RS_A in pFX2, it has yet to be established whether such a gene is present elsewhere on plasmids or the chromosome of the original lactococcal host. It has been suggested that although the presence of Pre is a requirement

for RS_A-mediated recombination, the host *rec* system may function to stimulate this recombination process (Gennaro et al. 1987).

Recently, a RS_A sequence has also been described in the *Lactobacillus plantarum* plasmid pLB4 (Bates and Gilbert 1989). A feature of further interest is that plasmids from different gram-positive genera isolated from different geographical origins share the same regions involved in genetic exchange, and also have similarly homologous regions for plasmid replication (de la Campa et al. 1990).

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