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pS86, A New Theta-Replicating Plasmid from *Enterococcus faecalis*

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Abstract. The complete nucleotide sequence of the small (5149 bp) and cryptic plasmid pS86 from *Enterococcus faecalis* ssp. *faecalis* S-86 has been determined. Sequence analysis revealed six putative open reading frames (ORFs) encoding polypeptides of 28.3, 11.5, 8.4, 65.1, 7.3, and 11.96 kDa each. Based on sequence similarity, two cassettes have been identified in pS86: ORF1 codes for the replication initiation protein (Rep); ORF4 codes for a putative mobilization protein that shows similarities to Mob/Pre proteins from plasmids of Gram-positive bacteria. No function could be assigned to the other putative ORFs found. According to our results, pS86 plasmid could use a theta-mode of replication, similar to the recently described theta-type replicons from pUCL287 (*Tetragenococcus halophila*) and pLA1 or pLA105 (*Lactobacillus acidophilus*) plasmids.

Enterococci are predominantly inhabitants of the intestine of humans and other animals. In spite of their ubiquity, they have seldom been reported as causative agents of serious infections, such as endocarditis and urinary tract infections. Among the different species of enterococci, *Enterococcus faecalis* has been genetically characterized to the greatest extent. A special interest in *E. faecalis* arose when it became clear that this species contains many plasmids and transposons and that at least some of them can be transferred conjugatively to other bacteria. Because of their preferential location in the intestinal tract and the abundance of extrachromosomal elements, enterococci can be considered as reservoirs of genetic information available to other (Gram-positive and even Gram-negative) intestinal bacteria [4, 13]. There have been several genetic studies to characterize enterococcal plasmids harboring genes encoding bacteriocins and/or hemolysins [21]. However, as no physiological role has been assigned yet to the cryptic plasmids, the information about them is scarce. Nowadays such studies are gaining interest, since increasing our knowledge on plasmid biology can provide useful tools for genetic improvement of *Enterococcus*.

This work deals with the genetic characterization of a small (5149-bp) cryptic plasmid found in *E. faecalis* S-86, whose DNA sequence has been elucidated. According to sequence similarities (both from DNA and the deduced translation products), the predicted products of two of the six ORFs found have been identified: one of them (ORF1) corresponds to replication initiation protein (Rep) and shows similarity with a new family of theta-replicons. A repeat sequence preceding ORF1 could contain the origin sequence essential for pS86 replication. ORF4 corresponds to the Mob protein, whose putative function could be the mobilization of this plasmid.

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Materials and Methods

Bacterial strains and growth conditions. *Enterococcus faecalis* S-86 from our laboratory collection was grown in brain heart infusion broth (BHI, Oxoid). *Escherichia coli* DH5α used for DNA recombinant experiments was grown in Luria broth (LB, Oxoid).

General methods, enzymes, reagents, and buffers. Plasmids from *E. coli* were isolated according to the alkaline lysis method [16]. Plasmids from *E. faecalis* were prepared by the procedure of Anderson and McKay [2] and further purified by CsCl/ethidium bromide density gradient centrifugation. Southern hybridization and other experimental procedures were carried out according to Sambrook et al. [16] unless otherwise mentioned. Detection of ssDNA was performed according to te Riele et al. [18]. Restriction endonucleases were purchased from Boehringer Mannheim or Gibco (BRL). T4 DNA ligase was supplied by Promega. In order to clone pS86, the plasmid was linearized with *Bgl*II and then ligated into the *Bam*HI site of plasmid vector pUC18. Recombinants were selected on media containing ampicillin (50 μg ml^{-1}) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

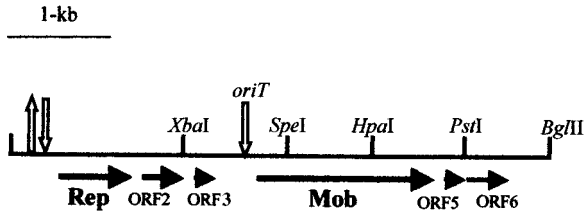

Fig. 1. Structural organization of pS86 plasmid. ORFs and location of some restriction enzymes with unique recognition site. Horizontal arrows indicate ORFs and the direction of transcription. Upward arrow indicates the site of directed repeat sequences. Downward arrows indicate sites of inverted repeat sequences. The probable transfer origin (*oriT*) is also shown.

DNA sequencing. The entire DNA sequence of both strands of pS86 was determined by the dideoxy-chain termination method by using a *Taq* Dye Deoxy Terminator Cycle Sequence kit (Applied Biosystems). DNA sequences were analyzed with the software of the University of Wisconsin Genetics Computer Group (GCG). Analysis of ORFs was carried out using the CODONPREFERENCE program; comparisons of sequences against EMBL Nucleic Database and the SwissProt Database were made by using FASTA, TFASTA, and BESTFIT programs. Protein alignments were made with the PILEUP and PRETTY programs. Protein secondary structures were predicted with PEPTIDESTRUCTURE and PLOTSTRUCTURE programs. The accession number of DNA sequence reported in this study is AJ223161 in the EMBL nucleotide sequence data base.

Results and Discussion

DNA sequence analysis of pS86. In order to investigate the structural organization of plasmid pS86 in detail, its complete DNA sequence was determined, starting rightwards from the unique *Bgl*II site. The complete sequence covered 5149 bp. Computer-assisted analysis of the sequenced region revealed six probable open reading frames (ORFs) covering 66.8% of the plasmid (Fig. 1).

The translation start point for each ORF was tentatively assigned by using several criteria: (i) the overall distribution of AT content in the third position, (ii) the codon usage of enterococci, and (iii) the potential for encoding proteins longer than 50 amino acids.

ORF1, located between nucleotides (nt) 500 and 1222, is able to encode a polypeptide of 240 amino acids residues with a molecular mass of 28.3 kDa and a theoretical pI of 7.7. This ORF is preceded by a typical consensus −10 region (TATAAT) and a potential −35 region (TTGACA).

The second reading frame (ORF2) mapped at position 1349–1651 (126 bp downstream of ORF1). The deduced translation product of this gene should be a 100-amino acid protein, with a predicted molecular mass of 11.5 kDa and a pI of 4.3.

The third ORF (ORF3) (nt 1775 to 1999) has a potential to encode a peptide of 74 amino acid residues with a molecular mass of 8.4 kDa and a pI of 10.7.

ORF4 extends from position 2430 to 4109, 932 bp downstream of ORF3, and it could encode a protein of 559 amino acids with a molecular mass of 65.1 kDa.

ORF5 (nt 4198 to 4398) is able to encode a polypeptide of 66 amino acids with a molecular mass of 7.3 kDa and a pI of 10.2.

The last reading frame found in pS86 (ORF6, nt 4431 to 4732), predicts a 103-amino acid protein, with an estimated size of 11.96 kDa and a pI of 9.6.

All protein-encoding regions have an ATG codon as putative initiation codon and are oriented in the same direction. A potential ribosomal-binding site (RBS) resembling those of some Gram-positives was identified upstream of each ORF. The RBS sequences for ORF1, ORF5 and 6 (GAGG), ORF3 (GGAG), and ORF2 and 4 (GGAGG) were located 12, 9, 8, 13, 9, and 8 nt upstream from initiation codons, respectively.

Consistent with our experimental data, the sequence of pS86 revealed unique sites for several restriction enzymes. Also, the sequence analysis revealed two series of direct repeats 5′ upstream of ORF1, identified as iterons whose function will be discussed latter on (Fig. 1).

Identification of DNA sequences involved in pS86 replication. Search for similarities between predicted products of pS86 putative ORFs and the protein sequences stored in databases revealed a high degree of similarity between the protein predicted for ORF1 and replication initiation proteins (Rep) encoded by plasmids pSK639 from *Staphylococcus epidermidis* (61.3% similarity, 48.8% identity; unpublished), pLA1 from *Lactobacillus acidophilus* (55.6% similarity, 43.3% identity) [20], pUCL287 from *Tetragenococcus halophila* (54.6 similarity, 39.5% identity) [3], and pLA105 from *Lb. acidophilus* (56.6% similarity, 39.5% identity) [9]. These results clearly suggest that ORF1 is involved in pS86 replication. Interestingly, plasmids pLA1, pLA105, and pUCL287 have been described as a new family of theta-type replicons, lacking homology with other known theta-type replicons from Gram-positive bacteria, such as pAMβ1 (*E. faecalis*), pIP501 (from *Streptococcus agalactiae*), and pUCL22 (from *Lactococcus lactis*) [3]. This result is interesting because among the family of small, multicopy, broad-host-range plasmids the most common type of replication is via single-stranded deoxyribonucleic acid (ssDNA) intermediates rolling-circle replication (RC).

In order to exclude the possibility that pS86 replicates via rolling circle, we performed Southern blot analyses according to te Riele et al. [18] to detect replication intermediates. By this procedure we could detect

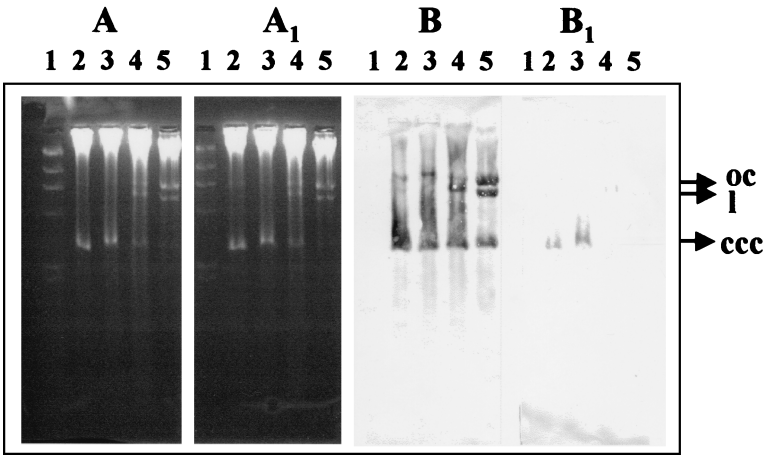

Fig. 2. Agarose gel electrophoresis showing supercoiled (cccDNA) pS86 plasmid. Lysates were prepared according to te Riele et al. [18] from strain S-86 after 4 h (lanes 2 and 4) and 8 h of growth (lanes 3 and 5). Lanes 4 and 5 show endonuclease S1-digested DNA. After electrophoresis, the DNA was transferred to nylon membranes with (A, B) or without previous denaturation (A_1, B_1). The filters were then hybridized (B, B_1) to the appropriate probes prepared from purified plasmid DNA. ccc indicates the position of supercoiled double-stranded plasmid forms; linear plasmid forms (1) observed in lanes 4 and 5 resulted from S1 nuclease digestion. Open circular forms are also observed (oc). Lane 1: standard molecular weigth, λ-*Hind*III.

only cccDNA, but no ssDNA derivatives of pS86 (Fig. 2). These results indicate, therefore, that pS86 does not exhibit rolling-circle replication.

The theta-replicating class A plasmids from Gram-positive bacteria contain, besides a *rep* gene (encoding a protein essential for the initiation of replication), an origin region (*ori*) with directly repeated sequences (iterons) which are the binding sites for Rep proteins and also are key elements for the control of plasmid replication [1]. Detailed analysis of the upstream *rep*-gene of pS86 showed an AT-rich zone that resembles the motif found in plasmids from *Lactobacillus* and other Gram-positive bacteria, that is otherwise essential to the initiation of replication. DNA homology also extends to regions upstream of the replication origin of pMMB1, another theta-replication plasmid from *E. faecium* [22] (positions 589 to 812, with a 75.33% identity; data not shown) whose significance is unknown. In fact, we found two series of iterons in the AT-rich region of pS86: the smaller series contains three repeats (6–10 bp) (position nt 223–251), and the larger one consists of a tandem of five direct repeats of 22 bp. Moreover, an outstanding inverted repeat (IR) of 54 nt is found encompassing the promoter region (−10 to −35) (position 423 to 476). Similar features have been described in Rep-region of *Pseudomonas* plasmid pPS10 [6].

Altogether, the data on the similarity of the Rep protein and the upper DNA sequences and the lack of ssDNA intermediates strongly suggest that pS86 undergoes theta replication. Theta-replicating plasmids are good candidates for the construction of cloning vectors for lactic acid bacteria because it is generally accepted that these plasmids are more stable than single-stranded ones.

Identification of the *mob* gene in pS86. Besides the Rep function, pS86 contains another cassette (ORF4) commonly found in other plasmids from Gram-positive bacteria, whose product shows the highest level of similarity with the family of mobilization (Mob) proteins from related Gram-positive bacteria: pLAB1000 from *Lb. hilgardii* (61.75% similarity, 50.23% identity) [8], pLC88 from *Lb. casei* (58.33% similarity, 45.17% identity, GenBank accession number U31333), pMV158 from *S. agalactiae* (59.53% similarity, 46.52% identity) [15], pTB913 from *Bacillus* sp. (50.92% similarity, 39.81% identity) [19], and pLA106 from *Lb. acidophilus* (62.4% similarity and 49.2% identity) [17].

The *mob* gene of pS86 predicts a protein of 559 amino acids (Mob86), slightly larger than the Mob proteins of the plasmids mentioned above, whose sizes range from 375 to 505 amino acid residues. The detailed analysis of their amino acid sequence showed that the highest similarities occurred within two regions: A (SNxDIDVxRSSHL**NYDL**V) and B (VHxDExTPHMH) located at the N-terminal part of proteins. The similarity is specially pronounced in the amino terminal moiety of the Mob proteins, where the predicted Mob86 protein showed values of similarity ca. 50% with the *Lactobacillus* proteins. Tentatively, these regions could correspond to the catalytic domains of Mob proteins, containing the conserved sequences NYDL (region A) and the consensus of region B, described respectively by Koonin and Ilyina [10] and Pansegrau et al. [14].

The mechanism of initiation of DNA transfer requires processing by nucleophilic attack (cleavage of the phosphodiester bond) of the Mob/Tra proteins on a specific DNA site. Alignment of the DNA sequences 5′ upstream of the *mob* gene in enterococcal plasmids pS86 with region *palD* of pMV158 (GenBank X15669) revealed the existence of palindromic sequence almost identical from nt 2340 to 2373 of pS86. This palindrome has been recently identified as the nicking site for Mob

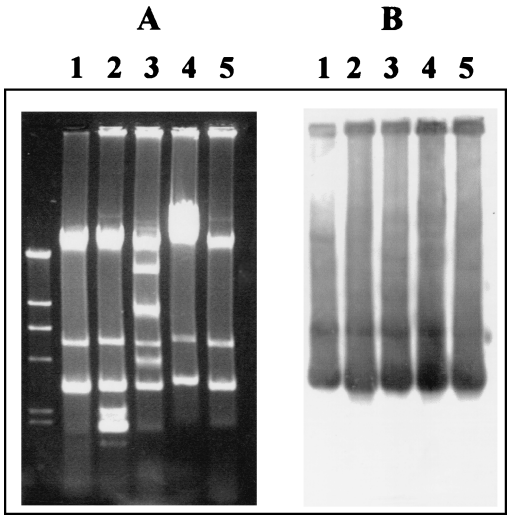

Fig. 3. Distribution of the pS86 plasmid in enterococci. (**A**) Ethidium bromide-stained gel showing the plasmid profile of different strains. (**B**) Hybridization analysis by using probes derived from *mob* gene of pS86. S-86 (lane 1), S-48 (lane 2), 39-5S (lane 3), EFS2 (lane 4), and EJ97 (lane 5). Standard molecular weight, λ-*Hind*III.

(*oriT*: TAGTTGxGTTAT) [7]. Comparison of this nucleotide sequence with upstream regions of the *mob* genes of different plasmids of Gram-positive bacteria stored in DNA database [GenBank m19465 (pUB110); U32369 (pBC16); Z11717 (pA1); M33531 (pLB4)] showed that identical or very similar sequences are also present. The existence of such inverted repeats in these plasmids suggests that they might act as a binding site for Mob proteins before DNA cleavage at *oriT*.

Other ORFs found in pS86. In relation to the other ORFs described, none of their predicted proteins showed significant homology/similarity to already known proteins from databases. On the basis of the deduced amino acid sequences, most of the gene products of these ORFs were predicted to be predominantly basic proteins (except for ORF2, which predicts a mostly acidic protein with a pI of 4.3). The product of ORF6 (a 103-amino acid protein, with an estimated size of 11.96 kDa and a pI of 9.6) deserves an additional commentary by its differential characteristics in relation to its amino acid composition, having an unexpected high proportion of hydrophobic amino acids: Ile (18 residues/mol), Leu (16 residues), Tyr (8 residues), Phe (7 residues), Val (6 residues).

Similarity of pS86 to other plasmids of *E. faecalis*. We have also carried out a comparative Southern blot analysis of pS86 with different cryptic plasmids present in other strains of *E. faecalis* from our collection. For this purpose, two different probes were obtained by PCR from *mob* and *rep* genes of pS86 to hybridize with DNA from enterococcal strains of known plasmid profiles, all of which harbored a plasmid of similar size to pS86. The results obtained demonstrated strong label with two plasmids of similar sizes (5.2 kb) found in the enterococcal strains S-48 (pMB3) [12], 39.5S (pPD4) [23], EFS2 [11], and EJ97 [5], confirming that this type of plasmids is widespread among enterococcal strains (Fig. 3). The virtual identity between these plasmids has been determined by PCR amplification with sequences from pS86 as primers. Furthermore, a new sequence has been deposited in GenBank (accession number D85392) having 99.8% identity with the *rep* gene and a part of *mob* from pS86 plasmid. The presence of these plasmids in enterococcal isolates of different and geographically distant locations suggests that there must be an efficient transfer mechanism, probably mediated by the *mob* genes. However, further research should throw light on the role of *mob* genes in mobility of this type of small cryptic plasmids.

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