Nucleotide sequence and structural organization of the small, broad-host-range plasmid pCI411 from *Leuconostoc lactis* 533

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The nucleotide sequence of the *Leuconostoc lactis* 533 cryptic plasmid pCI411 (2926 bp) was determined. Analysis revealed the presence of three open reading frames (ORFs). ORF 1 was capable of encoding a 24.9 kDa peptide which shared homology with the replication initiation protein (RepB) from a number of Gram-positive rolling circle plasmids. ORF 2 could encode a peptide of 6.6 kDa which was homologous to the RepC protein of the lactococcal plasmid pWV01. A function could not be assigned to ORF 3, which was capable of encoding a 12.1 kDa peptide. Transcription-translation analysis indicated the presence of three peptides of the predicted molecular masses. A putative double strand origin of replication (DSO) was identified which showed strong similarity with the DSO of a number of Gram-positive plasmids including pE194 from *Staphylococcus*. Structural analysis identified a number of direct and indirect repeats in addition to putative recombination-specific sites (RS_A and RS_B) in the non-coding region of pCI411. The observed characteristics suggest that this plasmid replicates using the rolling circle mechanism. pCI411, which could be introduced into *Leuconostoc*, *Lactococcus*, *Streptococcus*, *Lactobacillus* and *Bacillus* is the first plasmid from the genus *Leuconostoc* to be characterized in such detail.

Keywords: *Leuconostoc lactis*, cryptic plasmid

INTRODUCTION

The genus *Leuconostoc* represents a diverse group of heterofermentative lactic acid bacteria of considerable industrial importance. Traditionally, they have been used with other lactic acid bacteria in various food and wine fermentations where, in many cases, a number of beneficial qualities result from the dominance of *Leuconostoc* species (Mathieu *et al.*, 1993). For example, in dairy fermentations, the ability of some *Leuconostoc* species to produce diacetyl and carbon dioxide is important because of their contribution to flavour and eye-hole formation in Dutch-type cheeses. Like other lactic acid bacteria, many *Leuconostoc* species harbour one or more natural plasmids of various sizes. To date, phenotypes such as lactose utilization

(David *et al.*, 1992), citrate permease activity (David, 1992) and bacteriocin production and immunity (Hastings *et al.*, 1991) have been plasmid-linked in this genus but the majority of plasmids have no known function.

The study of small plasmids from a variety of bacteria has recently become a focus of research interest and in several cases this work has led to the development of families of vectors designed for specific purposes. The genetic organization and mode of replication of a number of small cryptic plasmids from some lactic acid bacteria have been described recently (Bates & Gilbert, 1989; Leer *et al.*, 1992; Xu *et al.*, 1991), and it is now known that these replicons are broadly similar to those from other Gram-positive (Gruss & Ehrich, 1989) and Gram-negative (Yang & McFadden, 1993) bacteria which replicate via single-stranded intermediates. They are collectively referred to as rolling circle (RC) plasmids. All RC plasmids harbour a gene encoding a plasmid replication initiation-termination protein (Rep), its target site, termed the double strand origin (DSO, formerly plus origin), and a

Abbreviations: SSO, single strand origin; DSO, double strand origin; RS, recombination-specific site; RC, rolling circle.

The GenBank accession number for the nucleotide sequence reported in this paper is L25529.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Reference/source
<i>Leuconostoc lactis</i>		
533	Wild-type strain	UCC culture collection
532	Wild-type strain	UCC culture collection
N2	Wild-type strain	UCC culture collection
NCW1	Wild-type strain	UCC culture collection
7-1	Wild-type strain	UCC culture collection
AH022	Plasmid-free derivative of 532	This study
AH211	AH022 containing pCI214 (Em ^R) and pCI411	This study
AH411	AH211 cured of pCI214	This study
AH431	AH022 containing pCI431	This study
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>		
X2	Wild-type strain	UCC culture collection
523	Wild-type strain	UCC culture collection
NCDO2028	Wild-type strain	UCC culture collection
<i>Leuconostoc paramesenteroides</i>		
NCDO1612	Wild-type strain	UCC culture collection
<i>Lactococcus lactis</i> subsp. <i>lactis</i>		
MG1614	Sm ^R , plasmid-free derivative of <i>L. lactis</i> subsp. <i>lactis</i> 712	Gasson (1983)
<i>Lactobacillus casei</i>		
20012	Wild-type strain	UCC culture collection
<i>Bacillus subtilis</i>		
DB104		Vehmaanperä (1989)
<i>Streptococcus salivarius</i> subsp. <i>thermophilus</i>		
054		Courtesy of A. Mercenier
<i>Escherichia coli</i>		
V517	Source of size reference plasmids	Macrina <i>et al.</i> (1978)
TG1	<i>supE</i> $\Delta(lac-proAB)$ <i>hsd</i> Δ 5 F' <i>traD</i> 36 (<i>r</i> _k ⁻ <i>m</i> _m ⁻) <i>proAB</i> ⁺ <i>lacI</i> ^q <i>lacZ</i> Δ M15	Sambrook <i>et al.</i> (1989)
HB101	F' <i>hsdS</i> 20 (<i>r</i> _b ⁻ <i>m</i> _b ⁻) <i>recA</i> 13 <i>ara-14</i> <i>proA</i> 2 <i>lacY</i> 1 <i>galK</i> 2 <i>rpsL</i> 20 (<i>Sm</i> ^R) <i>xyl-5</i> <i>mtl-1</i> <i>supE</i> 44	Boyer & Roulland-Dussoix (1969)
Plasmids		
pCI341	3.1 kb replication probe vector, Cm ^R	Hayes <i>et al.</i> (1990)
pNZ12	4.3 kb RC plasmid	Hayes <i>et al.</i> (1991)
pCI214	29 kb Em ^R plasmid	Caplice <i>et al.</i> (1987)
pCI411	2.7 kb Cryptic plasmid from <i>Leuc. lactis</i> 533	This study
pCI430	2.3 kb <i>HincII</i> fragment of pCI411 cloned in pCI341 (Rep ⁻)	This study
pCI431	<i>SstI</i> -linearized pCI411 cloned in pCI341 (Rep ⁺)	This study
pCI432	Excision of 1.4 kb <i>HpaI</i> - <i>SstI</i> region of pCI411 from pCI431 (Rep ⁻)	This study

specific sequence known as the single strand origin (SSO, formerly minus origin) which makes possible the conversion of ssDNA intermediates into dsDNA plasmid molecules. A different genetic organization is observed in plasmids which replicate using the theta mechanism (Hayes *et al.*, 1991). In the genus *Leuconostoc*, there have been no reports concerning the mode of replication and stability of plasmid molecules. In this paper, we describe the cloning and sequence analysis of the cryptic plasmid pCI411 (2926 bp) from *Leuconostoc lactis* 533 (type species). Its genetic organization and mode of replication is deduced, and its host range, stability and homology with other plasmids are examined.

METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. *Leuconostoc* strains were routinely subcultured in modified MRS (mMRS) broth (Difco) at 30 °C. *Lactococcus lactis* subsp. *lactis* MG1614 was grown at 30 °C in M17 medium (Terzaghi & Sandine, 1975) in which glucose (0.5%, w/v) replaced lactose (GM17). *Escherichia coli* strains were grown in L broth (Sambrook *et al.*, 1989) with shaking at 37 °C. *Bacillus subtilis* was grown in LB medium at 30 °C. Selective media contained antibiotics at the following concentrations: vancomycin, 100 µg ml⁻¹; chloramphenicol, 10 µg ml⁻¹ for *E. coli* and *Lactobacillus casei*, 5 µg ml⁻¹ for *Leuconostoc* strains, *Lc. lactis* subsp. *lactis* and *B. subtilis*, and

3.5 µg ml⁻¹ for *Streptococcus salivarius* subsp. *thermophilus*. Ampicillin (50 µg ml⁻¹) was used to maintain pUC19 in *E. coli*, and erythromycin (5 µg ml⁻¹) to maintain pCI214 in *Leuconostoc*.

DNA preparation and plasmid analysis. For rapid preparations of plasmid DNA from *Leuconostoc*, *Streptococcus* and *Lactococcus* strains the method of Anderson & McKay (1983) was used, with the following modifications for *Leuconostoc* species: cells resuspended in TES buffer [50 mM Tris/HCl (pH 8.0); 1 mM EDTA, 6.7% (w/v) sucrose] were treated with 15 mg lysozyme ml⁻¹ in 25 mM Tris/HCl (pH 8.0) at 37 °C for 15 min. After treatment with sodium dodecyl sulfate (20%, w/v in 50 mM Tris/HCl; 20 mM EDTA, pH 8.0), the suspension was left to clear for 20 min at 37 °C. Plasmid DNA was isolated from *Lb. casei* as described by Chassy & Flickinger (1987). For lysis of *E. coli* and *B. subtilis*, the method of Birnboim & Doly (1979) was used. Preparative amounts of plasmid DNA were obtained by scaling up these procedures followed by caesium chloride/ethidium bromide density gradient ultracentrifugation.

Restriction endonuclease analysis, molecular cloning techniques and nucleotide sequence analysis. Restriction endonucleases were purchased from Boehringer. DNA digestion and general cloning procedures were as outlined by Sambrook *et al.* (1989). Restriction analysis was performed as described previously (Coffey *et al.*, 1991). pCI411 DNA fragments generated using *Dra*I, *Hind*II, *Sst*I, *Hpa*I and *Hae*III were cloned into *E. coli* TG1 using pUC19. The nucleotide sequence was determined by sequencing double-stranded plasmid DNA in two orientations by the dideoxy chain termination method (Sanger *et al.*, 1977), using Sequenase version 2.0 (United States Biochemicals) and TaqTrack (Promega). Synthetic 17-mer DNA primers were prepared on a DNA synthesizer (Applied Biosystems). LaserGene (DNASTAR) and MicroGenie (Beckman) were used for computer-assisted sequence analysis.

Electroporation and transformation. *E. coli* electro-transformation was performed using a Gene Pulser apparatus and the conditions outlined in the manufacturer's instruction manual (Bio-Rad). *Leuconostoc* cultures were prepared for electro-transformation as follows. Cultures were grown overnight at 30 °C in mMRS broth supplemented with 40 mM DL-threonine and then diluted (1:50) in fresh broth and grown at 30 °C for 3–4 h. Cells were harvested by centrifugation at 4 °C, washed twice in ice-cold 10% (w/v) sucrose (Sigma), resuspended in 0.01 culture volume of ice cold 10% sucrose and held on ice until required. Up to 0.1 volume of plasmid DNA was thoroughly mixed with 100 µl of cell suspension in a chilled Gene Pulser cuvette (electrode separation of 2 mm). Cells were exposed to a single electric pulse (peak voltage 2.5 kV; capacitance 25 µF; resistance 200 Ω) which generated a peak field strength of 12.5 kV cm⁻¹. Immediately after delivery of the pulse, the cells were added to 5 ml mMRS broth and incubated at 30 °C for 2 h prior to spreading on mMRS agar plates containing the relevant antibiotics. Colonies were visible after 48 h. *Strep. salivarius* subsp. *thermophilus* 054 was electro-transformed as described by Slos *et al.* (1991), *Lc. lactis* subsp. *lactis* MG1614 as described previously (Coffey *et al.*, 1991), *Lb. casei* 20012 as described by Chassy & Flickinger (1987) and *B. subtilis* DB104 as described by Vehmaanperä (1989).

Southern hybridizations. After fractionation on 0.7% agarose gels, DNA was transferred to nitrocellulose filters by the method of Southern (1975). DNA was labelled using the enhanced-chemiluminescence (ECL) gene detection system (Amersham). Probe labelling, hybridization and washing steps were done according to the manufacturer's instructions.

Detection of ssDNA in whole-cell lysates of *Lc. lactis* and

***Leuconostoc*.** This was achieved using the method described by Leenhouts *et al.* (1991).

In vitro transcription-translation analysis. Protein products of pCI411 were identified from *in vitro* transcription-translation studies using a prokaryotic DNA-directed translation kit (Amersham) based on an S30 cell extract from *E. coli*. ³⁵S-labelled proteins were electrophoresed on 0.1% SDS/15% polyacrylamide gels. Gels were fixed in 7% (v/v) acetic acid, impregnated with Amplify (Amersham), dried and exposed to Hyperfilm MP autoradiography film (Amersham) for 48–96 h with an intensifying screen. ¹⁴C-methylated proteins (Amersham) were used as molecular mass markers.

RESULTS AND DISCUSSION

Isolation and characterization of pCI411

Leuconostoc lactis 533 is the type strain of this species (Garvie, 1986) and contains two plasmids of 12.5 kb and 2.9 kb, the latter designated pCI411. Total plasmid DNA was isolated from this strain and co-transformed with the 12.4 kb erythromycin resistance plasmid pCI214 (Caplice *et al.*, 1987) into the plasmid-free *Leuc. lactis* AHO22. Em^R transformants were obtained at a frequency of 3.52 × 10⁴ (µg DNA)⁻¹, of which 11% were shown to harbour pCI411. Repeated subculture at 37 °C was used to cure the marker plasmid from Em^R transformants. A representative derivative containing pCI411 alone was designated *Leuc. lactis* AH411. A map of pCI411 showing relevant restriction sites is presented in Fig. 1. In order to locate the genetic elements necessary for replication of pCI411, various constructs were generated by subcloning parts or all of the plasmid into the replication probe vector pCI341 (Hayes *et al.*, 1990), whose replication in *E. coli* HB101 is

Fig. 1. Map of pCI411 showing relevant restriction sites used for cloning and location of features derived from nucleotide sequence analysis: open reading frames (ORF), inverted repeat structures (IR) with ΔG values, direct repeats (DR), location of putative double strand origin (DSO) and recombination sequences (RS).

Fig. 2. Nucleotide sequence of plasmid pCI411 and predicted amino acid sequences of ORFs 1, 2 and 3. Boxed, recombination sequences RS_A and RS_B , putative double strand origin, -10 and -35 sequences, and ribosome-binding sites (RBS); horizontal solid arrows, direct repeats; horizontal dashed arrows, inverted repeats; *, stop codons.

supported by the pBR322 replicon, which is not functional in the *Leuconostoc* background. Thus the isolation of Cm^R transformants in *Leuc. lactis* AH022 implied that a functional pCI411 replicon was present (Table 1). Cloning of the entire plasmid using the unique *Sst*I site and subsequent transformation into *E. coli* resulted in the isolation of pCI431, which was the only recombinant

plasmid which could also replicate in *Leuconostoc* (Rep^+). When parts of pCI411 DNA were removed, generating the constructs pCI430 and pCI432 (Table 1 and Fig. 1), the ability to replicate was abolished, indicating that the region from *Hinc*II (934) to *Hinc*II (1442) and *Hpa*I (1442) to *Sst*I (2926) contained DNA which was essential for plasmid replication.

Fig. 3. Transcription-translation analysis of plasmids. Lanes: 1 and 7, ^{14}C -labelled molecular mass markers (in decreasing order of size) bovine serum albumin, ovalbumin, carbonic anhydrase and lysozyme; 2, DNA-free control; 3, pAT153 control (Amersham); 4, pCI341 vector (*cat* product); 5, pCI411 (the arrows indicate the positions of the transcription and translation products of ORFs 1, 2 and 3 with molecular masses of 24.9 kDa, 6.7 kDa and 12 kDa respectively); 6, pCI431, the chloramphenicol-resistant recombinant plasmid derived from pCI411 and pCI341, showing the products of all three ORFs of pCI411 and the *cat* product from pCI341.

Sequence analysis of pCI411

Plasmid pCI411 was sequenced to completion on both strands (Fig. 2). It comprises 2926 bp. Three significant open reading frames (ORFs) covering 38 % of the plasmid were identified. ORF1, from position 1638 to 2264, could encode a peptide of 208 amino acids with a molecular mass of 24.9 kDa. ORF2 begins at nucleotide 2260, overlaps the ORF1 codon by four base pairs and extends to position 2431. It had the capacity to encode a 56 amino acid peptide with a molecular mass of 6.7 kDa. ORF3 extends from position 2444 to 2761 and could encode a 105 amino acid peptide of molecular mass 12 kDa. Transcription-translation analysis of pCI411 indicated the presence of proteins of molecular masses corresponding to those predicted (Fig. 3). The three prominent bands in the sample containing pCI411 are also evident in the sample containing the pCI411-pCI341 recombinant plasmid, which also has the product of the pCI94 *cat* gene. The high level of background frequently encountered with the *in vitro* transcription-translation system is possibly due to the production of truncated derivatives of the larger gene products. All three ORFs have an ATG start codon, show the same orientation and are preceded by identifiable ribosome-binding sites at the consensus distance from the translational start reported for genes of Gram-positive bacteria (van de Guchte, 1991). A putative promoter region is located 261 bases upstream of ORF1. The putative -10 (TCTTAT) and the -35 regions (TGA-ACA) lie within a stem-loop structure with a calculated

free energy of binding, ΔG , of $-25 \text{ kcal mol}^{-1}$ ($-104.6 \text{ kJ mol}^{-1}$). It has been proposed that inverted repeat structures overlapping Rep promoters could constitute a target site for auto-regulation of Rep protein expression in the theta replicating plasmid pIP501 (Brantl & Behnke, 1992).

Comparison of the three pCI411-encoded proteins with the Swiss-Prot protein sequence databank (EMBL, Heidelberg, Germany) and NBRF-PIR (Washington DC, USA) revealed significant similarity with proteins from Gram-positive replicons. ORF1 showed 46.3 % homology to the replication initiation protein RepB from the *Lactobacillus plantarum* plasmid pLB4 (Bates & Gilbert, 1989). There was also significant homology to the replication initiation proteins of pADB201 of *Mycoplasma mycoides* (41.5 %; Bergemann *et al.*, 1989), pA1 from *Lb. plantarum* (28.7 %; Vujcic & Topisirovic, 1993) and pE194 from *Staphylococcus aureus* (22.2 %; Horinouchi & Weisblum, 1982). ORF2 showed 52.1 % similarity to ORF C of pWV01, believed to encode the repressor protein regulating synthesis of the replication initiation protein (Leenhouts *et al.*, 1991). There was no apparent similarity between ORF3 and other DNA or protein sequences in the data banks.

The homology of the translated peptide product of ORF1 to the replication initiation proteins of pLB4, pADB201, pA1 and pE194 argues in favour of pCI411 replicating by the RC mechanism. Where this system operates, a specific target site, the DSO, located upstream of the *rep* gene is nicked by the replication initiation protein. The sequence 5'-TACTACAACACCCCCCTATGT-3', which was homologous (one mismatch) to the DSO of RC plasmids pE194, pSH71, pLS1 and pWV01, was identified 440 bp upstream of the RepB start codon and is believed to be the DSO of pCI411. The observation that the plasmid construct pCI430 (Table 1 and Fig. 1), which lacks this region, could not replicate in *Leuconostoc* supports this proposal. ORF2 of pCI411, in addition to exhibiting strong homology to RepC of pWV01, is translationally coupled to ORF1 encoding RepB. In pWV01, pLB4 and pLS1 repressor proteins are also translationally coupled to the replication initiation proteins.

The SSO functions as the initiation site for DNA synthesis on the lagging strand. This site is normally located outside the minimal replicon and its deletion may cause a reduction in copy number, plasmid instability and accumulation of ssDNA intermediates (del Solar *et al.*, 1987). Different sequences representing the lagging strand initiation site have been characterized: *ssuA* (formerly *palA*, Gruss *et al.*, 1987), *ssuU* (formerly *palU*, Bron *et al.*, 1988), *ssuT* (formerly *palT*, Devine *et al.*, 1989) and a new type not belonging to these three groups which is highly conserved in plasmids like pLP4 and pLJ1 (Leer *et al.*, 1992). Typical SSOs are palindromic sequences of up to 200–300 bp in length with the potential to form hairpin secondary structures. In addition, all *ssuA*s harbour so-called RS_B sites at the 3' base of the stem, containing the 18 bp consensus sequence 5'-AAGTTTCTCGGCAT-AAA-3' (Gruss *et al.*, 1987). When pCI411 was examined for such characteristics, a similar sequence with one

mismatch was located at position 423–440 within a direct repeat and 37 bp downstream of an inverted repeat region (346–360:372–385, $\Delta G = -17 \text{ kcal mol}^{-1}$ ($-71.1 \text{ kJ mol}^{-1}$). However, unlike *ssaAs* this region does not contain the consensus sequence 5'-TAGCGT-3' (del Solar *et al.*, 1987) present in the loop of all *ssaAs*. As no other potential SSO motifs were identified on the plasmid, this region may be the SSO of pCI411.

Additional structural features of pCI411

Plasmid pCI411 contains two putative site-specific recombination sequences, RS_A and RS_B (Fig. 2). These sites have been studied in *Staph. aureus* plasmids, where they have been shown to be involved in stable cointegrate formation between small plasmids (Novick *et al.*, 1984; Gennaro *et al.*, 1987). The suggested 20 base RS_A site of pCI411 showed significant homology with that of pE194 (three mismatches); however, a genetic determinant for MOB (mobilization) which is involved in DNA nicking at the RS_A site in the pE194 system (Gennaro *et al.*, 1987) was not found on the *Leuconostoc* plasmid. The plasmid recombination/cointegration system mediated by the MOB-RS_A process is common in *Staphylococcus* and *Bacillus*, and the existence of MOB-RS_A complexes has been demonstrated in pLB4 from *Lb. plantarum* (Bates & Gilbert, 1989) and pLAB100 from *Lactobacillus hilgardii* (Josson *et al.*, 1990). Like pCI411, an RS_A region apparently lacking a MOB determinant has been described for plasmid pA1 of *Lb. plantarum* (Vujcic & Topisirovic, 1993) and lactococcal plasmid pFX2 (Xu *et al.*, 1991). In these instances it was speculated that, despite the absence of a MOB-determinant on the plasmid, the function may be encoded by the chromosome or a different plasmid in the original host, and hence cointegrate formation leading to conjugative mobilization may still occur. A number of imperfect direct repeats have been identified on pCI411 (Figs 1 and 2) and it is not known what their function, if any, might be. The potential role of all six of the stem and loop structures identified on pCI411 is also uncertain.

Detection of ssDNA intermediates during plasmid replication

Plasmids pCI411, and the control plasmids pNZ12 and pCI305, were assayed for their ability to produce ssDNA during replication. ssDNA was observed during replication of pCI411 as was also the case for pNZ12 which has previously been shown to produce ssDNA (Hayes *et al.*, 1991). pCI305, a small theta-replicating plasmid (Hayes *et al.*, 1991) employed as a negative control, did not generate ssDNA (data not shown). These observations strongly suggest that pCI411 replicates using the RC mechanism.

Host range and homology of pCI411 with *Leuconostoc* sp. plasmids

To determine whether pCI411 was homologous to other plasmids in various *Leuconostoc* species, pCI411 was labelled and probed against the plasmid profiles of seven

strains of *Leuc. lactis* and *Leuconostoc mesenteroides* (Table 1). Under the conditions of the experiment (high stringency) no hybridization was detected between pCI411 and the other *Leuconostoc* plasmids. pCI431, the Cm^R derivative of pCI411, was used to transform a number of genera by electroporation. Expression of this Cm^R gene, which is derived from the staphylococcal plasmid pC194 (Gruss & Ehrlich, 1989), was previously demonstrated in all the genera chosen for the pCI411 host-range studies. The pCI411 replicon was electroporated into, and shown to function in, *Leuconostoc mesenteroides* subsp. *mesenteroides* NCDO2028, *Leuconostoc paramesenteroides* NCDO1612, *Lc. lactis* subsp. *lactis* MG1614, *Strep. salivarius* subsp. *thermophilus* A054, *B. subtilis* DB104 and *Lb. casei* 20012. The replication functions did not operate in *E. coli* HB101, suggesting that pCI411 has a broad host range only among Gram-positive genera. It is, however, interesting that the DNA-directed transcription-translation system, which is based on an *E. coli* S30 extract, was apparently able to transcribe and translate all three ORFs of pCI411, while the plasmid could not be shown to replicate in this genus.

ACKNOWLEDGEMENTS

This work was supported by the European Community BRIDGE programme (contract BIOT-CT91-0263). We thank S. Foley for her valuable criticism of this manuscript and T. O'Sullivan for his assistance.

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Received 21 December 1993; revised 24 March 1994; accepted 31 March 1994.