Characterization of the theta-type plasmid pCD3.4 from *Carnobacterium divergens*, and modulation of its host range by RepA mutation

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The complete nucleotide sequence of the 3475 bp plasmid pCD3.4 from *Carnobacterium divergens* LV13, which encodes the bacteriocin divergicin A, was determined. Nucleotide sequence, deletion and complementation analyses revealed the presence of a *trans*-acting replication protein, RepA, and DNA sequences involved in plasmid replication and copy-number control. The DNA region preceding the *repA* gene probably contains the origin of replication. This sequence includes four and a half direct repeats (iterons) of 22 bp, to which RepA is thought to bind, and an AT-rich region containing a 12 bp repeat, at which initiation of DNA might occur. Further upstream of this sequence resides a fifth iteron required for optimal plasmid replication. The RepA protein shows homology to replication proteins of the pUCL287 subfamily of theta-type replicons. Two ORFs were found downstream of the *repA* gene that could be deleted without affecting replication and stability of the plasmid. pCD3.4 has a narrow host range, and could only be maintained in *Carnobacterium* spp.; however, a mutant of the plasmid was obtained that enabled the pCD3.4 replicon to replicate in *Enterococcus faecium*, but not in *Carnobacterium* spp. The mutation was located in the C-terminal region of the RepA protein, changing a proline into a serine. This is believed to be the first example of such plasmid-host-range modulation in Gram-positive bacteria.

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INTRODUCTION

Plasmids from lactic acid bacteria (LAB) have received considerable attention because they often contain important traits for fermentation, as well as genetic information for phage resistance and bacteriocin production. Furthermore, plasmids from a variety of LAB, especially from lactococci, have been characterized with the purpose of constructing cloning vectors to enhance the properties of LAB. Most of the small plasmids from LAB that are used as cloning vectors have rolling-circle-type replication. However, the structural and segregational instability of these plasmids for cloning DNA is well known (Kiewiet *et al.*, 1993b). In addition, the promiscuity of many rolling-circle replicating plasmids has made them relatively unsuitable for use as 'food-grade' cloning vectors in LAB. Therefore, the characterization of

the more stable theta-type replicating plasmids from LAB has received more attention.

Based on the mechanism of initiation of DNA replication in plasmid genomes, at least five classes of theta replicons have been identified (Bruand *et al.*, 1993; Meijer *et al.*, 1995). Class A plasmids depend on the presence of a plasmid-encoded replication protein (Rep). The host-encoded DNA polymerase I (PolI) is not required for replication of these plasmids. In addition to the *rep* gene, the replicon consists of an origin of replication (*ori*) that contains DNA iterons recognized by Rep, and an AT-rich region that generally contains repeats, as well as binding site(s), for the host-encoded DnaA proteins. Examples of class A plasmids in Gram-positive bacteria are pUCL22 (Frère *et al.*, 1993), pWVO2 (Kiewiet *et al.*, 1993a) and pCI305 (Hayes *et al.*, 1991), all of which are found in lactococci, and pLA103 from *Lactobacillus acidophilus* (Kanatani *et al.*, 1995) and pUCL287 from *Tetragenococcus halophilus* (Benachour *et al.*, 1997). Plasmids pSC101 and P1 from *Escherichia coli* are well-known examples of class A plasmids in Gram-negative bacteria (Abeles *et al.*, 1984; Churchward *et al.*, 1983). The broad-host-range streptococcal plasmids pAM β 1 (Bruand *et al.*, 1993) and pIP501 (Le Chatelier *et al.*, 1993) have been classified as class D plasmids. These plasmids require not

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Abbreviations: LAB, lactic acid bacteria; ssDNA, single-stranded DNA.

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only a Rep protein, but also PolI for replication. A novel type of replicon has been described for the *Bacillus subtilis* plasmid pLS20 (Class E, Meijer *et al.*, 1995); its minimal replicon does not contain an ORF, and it replicates in a PolI-mutated strain. Plasmid p256 from *Lactobacillus plantarum* was the first Rep-independent LAB plasmid to be identified (Sørvig *et al.*, 2005). An additional group of theta-replicating plasmids has DNA iterons within the coding sequence of the replication initiator protein, and members of this group are PolI-independent. Plasmid pLS32 (Tanaka & Ogura, 1998) of *Bacillus natto*, the enterococcal plasmids pAD1 (Weaver *et al.*, 1993), pCF10 (Hedberg *et al.*, 1996) and pPD1 (Fujimoto *et al.*, 1995), and the lactococcal plasmid pCI2000 (Kearney *et al.*, 2000), are examples of this family; however, this group of plasmids might be related to class A replicons (Kearney *et al.*, 2000).

To date, no plasmids from *Carnobacterium* spp. have been fully characterized. The production of the antibacterial peptide divergicin A from *Carnobacterium divergens* LV13 has been linked to the 3.4 kb plasmid pCD3.4, and its structural and immunity genes have been cloned and sequenced (Worobo *et al.*, 1995). This paper describes the entire nucleotide sequence of pCD3.4. Here, we report on the genetic identification of elements involved in pCD3.4 replication, and show that its replicon is of Class A, and that its narrow host range is influenced by the Rep protein. A possible explanation for this host-range modulation is discussed.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
<i>E. coli</i> MH1	MC1061 derivative; <i>araD139 lacX74 galU galK hsr hsm1 strA</i>	Casadaban & Cohen (1980)
<i>E. coli</i> BL21(DE3)	F ⁻ <i>ompT hsdS_B (r_B⁻ m_B⁻) gal dcm</i> (DE3)	Studier & Moffat (1986)
<i>C. divergens</i> LV13		Worobo <i>et al.</i> (1995)
<i>C. maltaromaticum</i> UAL26		Laboratory collection
<i>C. maltaromaticum</i> LV17C		Ahn & Stiles (1990)
<i>Lc. lactis</i> MG1363		Gasson (1983)
<i>Ent. faecalis</i> ATCC 19433		ATCC
<i>Ent. faecium</i> BFE900		Franz <i>et al.</i> (1999)
<i>Leu. gelidum</i> UAL187-13		Hastings & Stiles (1991)
<i>Lb. casei</i> ATCC 393		ATCC
Plasmids		
pUC118	<i>lacZ'</i> , 3.2 kb, Ap ^r	Vieira & Messing (1987)
pUC7e	pUC7 containing 1.0 kb Em ^r gene of pE194 in <i>Pst</i> I site, 3.7 kb	UofG*
pGS30	pUC7 containing 1.0 kb Cm ^r gene of pC194 in <i>Pst</i> I site, 3.7 kb	UofG
pUC118e	pUC118 containing 1.0 kb Em ^r gene, 4.2 kb	This study
pUC118-C	pUC118 containing 1.0 kb Cm ^r gene, 4.2 kb	This study
pMG36e	3.6 kb, Em ^r	van de Guchte <i>et al.</i> (1989)
pT713	2.8 kb, Ap ^r , T7-polymerase expression vector	Tabor & Richardson (1985)
pTCD1	pT713 containing 2.2 kb <i>Sac</i> I- <i>Eco</i> RV fragment, 5.0 kb	This study
pCD3.4	3.475 kb	Worobo <i>et al.</i> (1995)
pCD5	3.4 kb <i>Eco</i> RV fragment ligated to 1.0 kb Em ^r gene	This study
pCD6	2.1 kb <i>Mbo</i> I fragment ligated to 1.0 kb Em ^r gene	This study
pCD12	1.0 kb <i>Hin</i> PI- <i>Ava</i> II fragment ligated to 1.0 kb Em ^r gene	This study
pCD14	pMG36e containing 0.7 kb <i>repA</i> gene, 4.3 kb	This study
pCD15	pUC118-C containing 1.5 kb <i>Mbo</i> I- <i>Eco</i> RI fragment, 5.7 kb	This study
pCD16	2.0 kb <i>Ava</i> II fragment ligated to 1.0 kb Em ^r gene	This study
pCD17	1.4 kb <i>Hind</i> III- <i>Ava</i> II fragment ligated to 1.0 kb Em ^r gene	This study
pCD18	1.4 kb <i>Hin</i> PI fragment ligated to 1.0 kb Em ^r gene	This study
pCD51	pCD5 containing <i>repA</i> mutation, 4.4 kb	This study
pCD52	pCD5 containing <i>repA</i> mutation, 4.4 kb	This study
pCD70	pUC118e containing 1.4 kb <i>Hin</i> PI fragment, 5.6 kb	This study
pCD71	pUC118e containing 1.3 kb <i>Sac</i> I- <i>Hin</i> PI fragment, 5.5 kb	This study

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in TY broth (Rotlander & Trautner, 1970) at 37 °C; carnobacteria, enterococci and leuconostocs were grown in APT broth (All Purpose Tween; Difco) at 25, 30 and 30 °C, respectively. *Lactococcus lactis* was grown in glucose-M17 broth (Terzaghi & Sandine, 1975) at 30 °C, and lactobacilli were grown in MRS broth (De Man *et al.*, 1960) at 37 °C. When appropriate, antibiotics were added to the media at the following selective concentrations: erythromycin (200 µg ml⁻¹), chloramphenicol (25 µg ml⁻¹) and ampicillin (150 µg ml⁻¹) for *E. coli*; erythromycin (5 µg ml⁻¹) for *Carnobacterium maltaromaticum*, *Lactobacillus casei*, *Leuconostoc gelidum* and *Lc. lactis*; chloramphenicol (5 µg ml⁻¹) for *C. maltaromaticum*. Erythromycin was used at 50 and 150 µg ml⁻¹ for *Enterococcus faecalis* and *Enterococcus faecium*, respectively.

DNA manipulations and transformations. Cloning and DNA manipulations were performed as described by Sambrook *et al.* (1989). Enzymes used for molecular cloning were obtained from Invitrogen Life Technologies, and used as specified by the manufacturer. Plasmid DNA from *E. coli* was isolated as described by Birnboim & Doly (1979); with some modifications, the same method was used to isolate plasmid DNA from LAB (van Belkum & Stiles, 1995). Nucleotide sequencing was based on the method of Sanger *et al.* (1977), and carried out in a Perkin-Elmer ABI-Prism DNA sequencer, with fluorescent chain terminators. Electroporation of *Lc. lactis* was performed as described by Holo & Nes (1989). For transformation of other LAB, cells were grown in APT or MRS medium supplemented with 2% (w/v) glycine. Exponentially growing cells were harvested, and washed twice with ice-cold water, and twice with ice-cold electroporation buffer (0.5 M sucrose, 10%, v/v,

glycerol, 1 mM MgCl₂, 5 mM potassium phosphate buffer, pH 6), concentrated 100-fold in the same buffer, divided into 50 µl portions, and stored at -70 °C. Electroporation was carried out as described by van Belkum & Stiles (1995), using a Gene-Pulser instrument (Bio-Rad). Competent *E. coli* cells were transformed by the method of Mandel & Higa (1970).

Detection of single-stranded DNA. For detection of single-stranded DNA (ssDNA) in *C. divergens* LV13, the method described by Leenhouts *et al.* (1991) was used. Non-radioactive probes were made with a random-primed labelling and detection kit (Boehringer Mannheim), and used in Southern hybridization.

Construction of plasmids. Deletion derivatives of plasmid pCD3.4 were made using the erythromycin gene of pUC7e as a selection marker (Fig. 1). The 1 kb *Bam*HI fragment containing the erythromycin gene from pUC7e was isolated, treated with Klenow enzyme, and ligated into pCD3.4 that had been cut by *Eco*RV. The resulting plasmid, pCD5, contains a deletion in the promoter region of the divergicin A operon. Plasmid pCD6 was constructed by ligating pCD3.4, which had been digested with *Mbo*I, with the 1 kb *Bam*HI fragment containing the erythromycin gene from pUC7e. Plasmids pCD12, pCD16, pCD17 and pCD18 were constructed by ligating the 1 kb *Bam*HI fragment from pUC7e with pCD3.4 cut with *Ava*II and *Hin*PI, *Ava*II, *Ava*II and *Hind*III, or *Hin*PI, respectively, after treatment with Klenow enzyme. To investigate further the exact size of the pCD3.4 replicon, plasmids pCD70 and pCD71 were made in *E. coli* as follows: a 1 kb *Sal*I fragment containing the erythromycin gene from pUC7e was cloned into pUC118, giving

Fig. 1. Schematic representation of plasmid pCD3.4 and its subclones. The location and direction of transcription of *dvnA*, *dviA*, *repA*, *orf-1* and *orf-2*, and the positions of some restriction sites, are shown. The ability of various deletion derivatives to replicate in *C. maltaromaticum* UAL26, and their segregational stability, are indicated. The promoters P32 and T7, used to express some of the subclones, and the direction of their transcription, are shown. ND, Not done.

pUC118e; the 1.4 kb *Hin*PI fragment from pCD3.4 was blunt-ended by Klenow enzyme, and ligated into the *Sma*I site of pUC118e, resulting in plasmid pCD70; a 65 bp deletion in the pCD3.4 replicon of pCD70 was made by digesting this plasmid with *Sac*I, followed by self-ligation of the remaining fragment, giving plasmid pCD71. Plasmids pCD14 and pCD15 were constructed for the complementation experiment with the *repA* gene. Using primers MB63 and MB64 containing the restriction sites *Xba*I and *Kpn*I (underlined), respectively, the *repA* gene of pCD3.4 was amplified by PCR. The nucleotide sequences of the two primers were as follows: MB63, 5'-ATATTCTAGATGTGAGGGGAGGTCGAAA-3'; MB64, 5'-TAT-AGGTACCTTTTATTCGTCAGTCGTG-3'. The resulting PCR product was cloned into the *Xba*I and *Kpn*I restriction sites of pMG36e, giving plasmid pCD14. Plasmid pUC118-C was constructed by cloning the 1 kb chloramphenicol gene of pGS30 into the *Sal*I site of pUC118. The 1.5 kb *Mbo*I-*Eco*RI fragment of pCD3.4 was then cloned into the *Bam*HI and *Eco*RI sites of pUC118-C, resulting in plasmid pCD15. To overexpress genes of interest, plasmid pCTD1 was constructed by cloning the 2.2 kb *Sac*I-*Eco*RV fragment of pCD3.4 into the *Sac*I and *Sma*I restriction sites of the expression vector pT713.

Plasmid stability. To investigate the segregational stability of pCD3.4 derivatives, cultures of *C. maltaromaticum* UAL26 containing these plasmids were first grown in APT containing erythromycin as selective pressure at 25 °C. After achieving full growth, the cultures were diluted 10^{-6} in non-selective medium, and grown for an additional 24 h until full growth (equivalent of 20 generations). The fully grown cultures were diluted again 10^{-6} in non-selective medium. This was repeated until the cultures had grown for 100 generations in the absence of selective pressure. To determine the percentage of cells that were resistant to the antibiotic, cells from fully grown cultures after each dilution step were plated on APT agar plates without antibiotic, and grown overnight at 25 °C. The next day, 100 colonies from each culture were stabbed onto APT agar plates with and without erythromycin, and grown for an additional 24 h to determine the percentage of cells that had lost the plasmid.

Overexpression of proteins in *E. coli* by T7 RNA polymerase. Proteins in cultures of *E. coli* BL21(DE3) were overexpressed, as described by van Belkum *et al.* (1997).

RESULTS AND DISCUSSION

Nucleotide sequence analysis

Part of plasmid pCD3.4 had previously been sequenced (coordinates 1–581), and it revealed the presence of the divergicin A operon consisting of the divergicin A structural gene (*dvnA*), its cognate immunity gene (*dviA*), and a potential *rho*-independent terminator (Worobo *et al.*, 1995). Plasmid pCD3.4 has now been completely sequenced, and consists of 3475 bp, with a G + C content of 31.3 mol%. Analysis of the sequence revealed the presence of three additional ORFs (Fig. 1). The first ORF, *repA*, could encode a protein of 238 aa that shows homology with replication proteins from the pUCL287 family of theta-type replicating plasmids, such as Rep1 from pIP1629 of *Staphylococcus epidermidis* (46 % identity) (Aubert *et al.*, 1998), Rep from pS86 of *Ent. faecalis* (66 % identity) (Martínez-Bueno *et al.*, 2000), RepA from pLA103 of *Lactobacillus acidophilus* (38 % identity) (Kanatani *et al.*, 1995), RepA from pLKS of *Lactobacillus plantarum* (34 % identity) (Eguchi *et al.*, 2000), and RepA from pUCL287 from *Tetragenococcus halophilus* (33 % identity) (Benachour *et al.*, 1997). Plasmid pUCL287 replicates by a bidirectional theta mechanism, and is an example of a class A replicon (Benachour *et al.*, 1995, 1997). A putative promoter and a RBS were located upstream of the *repA* gene (Fig. 2). *orf-1* and *orf-2* (Fig. 1) were found downstream of *repA*, and could encode proteins of 185 and 94 aa, respectively. RBSs precede both ORFs, but no homology was found in the database with their predicted proteins. No putative promoters were found immediately upstream of these two ORFs, indicating that *orf-1* and *orf-2* might be co-expressed with *repA*. A putative promoter (coordinates 3454–8), with TTGGAT as the –35 region, and TATCTT as the –10 region, was located upstream of *dvnA*. Primer extension analysis of *dvnA* confirmed the presence of this promoter structure (J. K. McCormick & L. Saucier, unpublished data).

Fig. 2. Nucleotide sequence of the putative origin of replication and the 5' end of *repA*. The deduced amino acid sequence for the N-terminal part of RepA is shown above the nucleotide sequence. The DNA iteron (DR1) and the 12 bp direct repeats (DR2) are indicated by arrows. The putative –35 and –10 promoter regions, and a possible RBS, are indicated, as well as the recognition sites for restriction enzymes *Hin*PI and *Sac*I (bold italics). The GC clusters are double underlined, and a nucleotide sequence identical to the 11 bp repeats found in pS86 (Martínez-Bueno *et al.*, 2000) is indicated by a dashed line; the sequence corresponds to nt 1051–1550 of accession no. DQ087597.

Like many Class A replicons, two types of direct repeats were found upstream of the putative promoter sequence of *repA*. A 22 bp iteron is repeated 5.5 times. The first (coordinates 1177–1198) and second repeats (coordinates 1327–1348) are separated by a 128 bp sequence that contains a 56 bp AT-rich region flanked by GC clusters, and carries a 12 bp direct repeat (coordinates 1249–1272) (Fig. 2). It is proposed that the AT-rich region is the site where the two DNA strands separate during replication initiation, and that the GC clusters act as clamps to limit melting of the strands in the direction of the direct repeats (Kim & Meyer, 1991). A DnaA box was not found on pCD3.4, indicating that this host-encoded protein might not be involved in plasmid replication. The iterons of pS86 and pCD3.4 are very similar: 15 out of 22 nucleotides are identical, including the first three and the last nine nucleotides of the repeats (Martínez-Bueno *et al.*, 2000). The fact that the Rep proteins and iterons of pS86 and pCD3.4 share significant similarity is not surprising, assuming that these replication proteins recognize and interact with the iterons to regulate plasmid replication. The two 12 bp repeats in the AT-rich region of pCD3.4 differ from the two 11 bp repeats in pS86, although an 11 bp sequence (5'-ATATATATTTT-3'), identical to the 11 bp repeats of pS86, was found adjacent to the 12 bp repeats of pCD3.4. The significance of this finding is not known.

Several theta-type replicons, including that of pS86, contain one or more inverted repeats that overlap the promoter region of the replication gene (Benachour *et al.*, 1997; Foley *et al.*, 1996; Martínez-Bueno *et al.*, 2000). In pCI305, it has been shown that the replication protein binds to the inverted repeat, probably to autoregulate its own expression (Foley *et al.*, 1996). However, such inverted repeats were not observed in the promoter region of *repA* of pCD3.4, suggesting a different mechanism of regulation.

The 2.2 kb *SacI*–*EcoRV* fragment of pCD3.4 containing *repA*, *orf-1* and *orf-2* was cloned behind the inducible T7 promoter of pT713, resulting in pTCD1. Cell lysates of induced or uninduced *E. coli* BL21(DE3) cultures containing pTCD1 were examined using Tricine SDS-PAGE. Expression of the cloned insert of pTCD1 after induction with IPTG resulted in the production of three proteins of 28, 22 and 12 kDa (Fig. 3). This result is in good agreement with the predicted molecular masses of 28.0, 21.9 and 11.5 kDa for RepA, Orf-1 and Orf-2, respectively, and confirms the presence of the genes encoding these proteins.

Detection of ssDNA

Whole-cell lysates of LV13 transformed with plasmid pMG36e were treated or not treated with S1 nuclease, and hybridized with pCD3.4 or pMG36e DNA, to determine whether pCD3.4 produces ssDNA, using pMG36e as a positive control; pMG36e is a derivative of the rolling-circle plasmid pWVO1 (van de Guchte *et al.*, 1989). ssDNA was

Fig. 3. Overexpression of the gene products of *repA*, *orf-1* and *orf-2* in *E. coli* by the T7 promoter. Lanes 1 and 2, lysates of *E. coli* BL21(DE3) containing pTCD1 and pT713, respectively. Lane 3, low-molecular-mass marker (Bio-Rad). The positions of RepA, Orf-1 and Orf-2 are shown on the left, and the sizes of the molecular standard (kDa) are shown on the right.

detected from pMG36e, but not from pCD3.4 (Fig. 4), providing further evidence that pCD3.4 belongs to the theta-type family of replicons.

Fig. 4. Hybridization of plasmid DNA in whole-cell lysates of *C. maltaromaticum* LV13 containing pMG36e, using pMG36e (A) and pCD3.4 (B) as probes. DNA from the whole-cell lysates was treated (+) or not treated (–) with S1 nuclease. OC, CC and SS indicate the positions of open circular, covalently closed circular and single-stranded DNA, respectively.

Identification of the replication and copy-number control regions of pCD3.4

Fragments of pCD3.4 were ligated with a 1 kb DNA fragment containing the erythromycin-resistance marker, and transformed into *C. maltaromaticum* UAL26 (Fig. 1). The results show that the smallest fragment from pCD3.4 able to transform UAL26 was a 1 kb *HinPI*–*AvaII* fragment in pCD12. Apparently, the only ORF on pCD3.4 required for replication is *repA*. Two plasmids, pCD14 and pCD15, were constructed to assess the function of RepA in replication. Plasmid pCD14 contains the *repA* gene cloned behind the constitutive lactococcal P32 promoter of the shuttle vector pMG36e, with erythromycin as the selection marker. In pCD15, a 1.5 kb *MboI*–*EcoRI* fragment containing the putative origin of replication of pCD3.4, and a truncated *repA* gene, was cloned into the chloramphenicol-encoding *E. coli* vector pUC118-C. Chloramphenicol-resistant transformants of *C. maltaromaticum* UAL26 could not be obtained after electroporation with pCD15; however, when *C. maltaromaticum* UAL26 contained pCD14, this strain could be transformed with pCD15. Plasmid profile analysis showed that these transformants contained two independent plasmids (pCD14 and 15), and not a product arising from integration of pCD14 into pCD15 by homologous recombination at *repA* (data not shown). This shows that RepA complements *in trans* the replication of the pCD3.4 replicon.

Two plasmids were made based on pUC118e to investigate whether the 65 bp *HinPI*–*SacI* fragment, which contains the two 12 bp repeats in the AT-rich region, is essential for replication of pCD3.4 in *Carnobacterium* spp. Plasmid pUC118e replicates in *E. coli*, but not in *Carnobacterium* spp. Cloning the 1.4 kb *HinPI* fragment from pCD3.4 into pUC118e created plasmid pCD70, and plasmid pCD71 was constructed by deleting the 65 bp *HinPI*–*SacI* fragment from pCD70. Electroporation of *C. maltaromaticum* UAL26 with pCD70 or pCD71, and selecting for erythromycin resistance, resulted in transformants containing pCD70 only. This result indicates that the 65 bp *HinPI*–*SacI* segment is necessary for replication. Deletion of two of the four 11 bp repeats in pUCL287 also abolished plasmid replication, indicating that the presence of these types of repeats is essential for replication (Benachour *et al.*, 1997).

To search for the region required for stable maintenance of the pCD replicon, *C. maltaromaticum* UAL26 cells containing pCD5, pCD16, pCD17 or pCD18 were grown in the absence of erythromycin. Erythromycin resistance of *C. maltaromaticum* UAL26 transformed with pCD5, pCD16 or pCD17 was not lost after 100 generations (data not shown), indicating that *orf-1*, *orf-2*, *dvnA* and *dviA* do not play a role in plasmid maintenance. However, less than 1 % of UAL26 transformed with pCD18 showed erythromycin resistance after 20 generations. Plasmid DNA was not detected when plasmid DNA was isolated from cells that had lost their erythromycin resistance, indicating that the erythromycin-sensitive cells had lost pCD18. Agarose gel electrophoresis of

the plasmid isolated from *C. maltaromaticum* UAL26 containing pCD18, which was grown in the presence of the antibiotic, showed little DNA compared with cells containing pCD5, pCD16 or pCD17 (data not shown). No difference was observed in the plasmid copy number between pCD5, pCD16 and pCD17 (data not shown). These results indicate that deleting a 0.4 kb *HindIII*–*HinPI* DNA region containing the first 22 bp repeat of pCD3.4 reduces the copy number, and affects the stability of the pCD replicon. It is not uncommon for theta-type plasmids to contain auxiliary iterons. The iteron is the target of the replication initiator protein, and may be important in the control of plasmid copy number. In plasmid P1, deleting these extra copies of iterons increases the copy number of the plasmid (Pal *et al.*, 1986). In contrast, removing the region in pCD3.4 that contains the extra 22 bp repeat actually reduces the copy number. This was confirmed by the low yield of plasmid pCD12, a plasmid that also lacks the 0.4 kb *HindIII*–*HinPI* DNA region, isolated from UAL26 (data not shown). The fact that plasmids such as pS86 and pUCL287 contain one or more inverted repeats overlapping the promoter of the replication region, and that they do not seem to have auxiliary iterons, suggests that the copy number of pCD3.4 is regulated differently to these other plasmids. Further work needs to be done to show that the copy number and stability of pCD3.4 indeed depend on this auxiliary iteron, and not on something else that is located on this 0.4 kb *HindIII*–*HinPI* fragment.

Strict host range of pCD3.4, and its modification by mutation in RepA

To determine whether pCD3.4 can replicate in other organisms, plasmid pCD5 was used to transform a variety of bacterial strains. The erythromycin gene in pCD5 was inserted in such a way that it inactivated the divergicin operon by deleting the promoter region upstream of the divergicin A structural gene. Plasmid pCD5 could be transformed into *C. maltaromaticum* strains LV17C and UAL26, but transformants could not be obtained with *E. coli* MH1, *Lc. lactis* MG1363, *Ent. faecalis* ATCC 19433, *Leu. gelidium* UAL187-13 or *Lb. casei* ATCC 393. These results suggest that pCD3.4 has a narrow host range, and may only replicate in *Carnobacterium* spp.

Interestingly, after 2 days of incubation on APT agar plates, two erythromycin-resistant colonies were obtained when plasmid pCD5 was transformed into the plasmid-less strain *Ent. faecium* BFE900; both colonies harboured a pCD5-type plasmid. Transformation of BFE900 with the broad-host-range plasmid pMG36e as a positive control yielded more than 10^3 transformants ($\mu\text{g DNA}$)⁻¹. These results indicate that although the two BFE900 colonies were genuine transformants, pCD5 cannot readily be transformed into BFE900. Plasmids extracted from the two transformants, named pCD51 and pCD52, were used to transform *C. maltaromaticum* UAL26. pCD5 was used as a positive control for transforming UAL26. More than 10^4 transformants ($\mu\text{g DNA}$)⁻¹ were obtained after transformation with

pCD5, whereas no transformants were obtained with pCD51 and pCD52. When pCD51 and pCD52 were used to electroporate BFE900, more than 10^3 transformants ($\mu\text{g DNA}$)⁻¹ were obtained with both plasmids, but no transformants were observed after 2 days of incubation following electroporation with plasmid pCD5. These results demonstrate that plasmids pCD51 and pCD52 underwent changes that altered the host specificity of the pCD derivative. The replication region of both pCD51 and pCD52 was sequenced at the nucleotide level. No mutation was found in the region upstream of the RepA gene, including the DNA iterons and other sequences essential for replication of the pCD replicon. However, a single amino acid substitution was observed at position 232 inside the RepA protein encoded by pCD51 and pCD52. In both plasmids, the CCA codon for proline was changed into a TCA codon for serine. The fact that this mutation was identical in two separate *Ent. faecium* transformants most likely confirms that this change in RepA changed the host range of the pCD3.4 replicon. Attempts to transform *Ent. faecalis* ATCC 19433 with pCD51 and pCD52 were unsuccessful. Apparently, this amino acid substitution in the C-terminal part of the RepA protein enabled the plasmid to change its narrow host range for replication from *C. maltaromaticum* to *Ent. faecium*. Modulation of the host range of a plasmid by mutations in the replication protein has also been described for pPS10 of *Pseudomonas savastanoi*. Plasmid pPS10 cannot establish itself in *E. coli* at 37 °C, and replicates poorly in this host at 30 °C. However, mutations in RepA of pPS10 have been obtained that result in transformants of *E. coli* that grow at 37 °C (Fernández-Tresguerres *et al.*, 1995; Maestro *et al.*, 2003). An enhanced interaction of the host-encoded DnaA protein with these mutant RepA proteins is observed (Maestro *et al.*, 2003). Furthermore, pPS10 can also be established in *E. coli* by a chromosomal mutation of the *dnaA* gene, giving further evidence that interaction between host-encoded DnaA and plasmid-encoded RepA influences the host range of pPS10 (Maestro *et al.*, 2002). It is unclear whether such a mechanism is also responsible for the ability of the RepA(P232S) mutant of pCD3.4 to replicate in *Ent. faecium* and not in *C. maltaromaticum*. The change of the host range of pCD3.4 by this *repA* mutation might be explained by assuming that the RepA protein of pCD3.4 interacts in a similar manner with a host-encoded protein. This interaction could take place with the C-terminal domain of RepA, where the mutation is located; however, the possibility that the mutation induces a conformational change elsewhere in the Rep protein cannot be ruled out. The fact that a DnaA box was not found in pCD3.4 could indicate that other host-encoded proteins are involved in the interaction with RepA. It has been shown that plasmid replication proteins can interact with other host-encoded replication proteins, such as DnaB helicase and DnaG primase (Abhyankar *et al.*, 2004; Datta *et al.*, 1999; Jiang *et al.*, 2003). The finding that pCD3.4 has a very narrow host range indicates that host factors may play a role in the host-range specificity of this plasmid. These results might form a basis to develop cloning vectors based

on pCD3.4 that are tailored towards specific bacterial strains.

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