pBLA8, from *Brevibacterium linens*, belongs to a Gram-positive subfamily of ColE2-related plasmids

Véronique Leret, Annie Trautwetter, Alain Rincé and Carlos Blanco

Author for correspondence: Carlos Blanco. Tel: +33 2 99 28 61 40. Fax: +33 2 99 28 61 40.
e-mail: Carlos.Blanco@univ-rennes1.fr

Membranes et
Osmorégulation, UPRES-A
6026, Université Rennes I,
Avenue du Général
Leclerc, 35042 Rennes
Cedex, France

A 3.1 kb DNA fragment from pBLA8, a *Brevibacterium linens* cryptic plasmid, containing all the information required for autonomous replication was cloned and sequenced. Using deletion analysis, the fragment essential and sufficient for autonomous replication was delimited to 1.5 kb. This fragment is characterized by the presence of an *ori* site located upstream of an operon encoding two proteins, RepA and RepB, both essential for replication. Based on structural similarities and a strong conservation of *ori*, RepA and RepB, pBLA8 was assigned to a new subfamily of the ColE2 plasmid family. This subfamily is distinguished by the requirement for two Rep proteins and the location of an *ori* site upstream of the *repAB* operon. RepA is thought to encode primase activity, whereas RepB could be a DNA-binding protein. An *Escherichia coli*-*B. linens* shuttle vector, derived from pBLA8, was constructed. Its host spectrum was extended to *Arthrobacter* species.

Keywords: *Brevibacterium linens*, plasmid replication, ColE2 related plasmids, shuttle vector

INTRODUCTION

The genus *Brevibacterium* includes irregular non-spore-forming, Gram-positive, strictly aerobic rods, and belongs to the same group of coryneform bacteria as the genera *Corynebacterium*, *Arthrobacter*, *Cellulomonas*, *Curtobacterium* and *Microbacterium*. Within this group, *Corynebacterium* is distinct from the others with regard to its cell wall and lipid composition that make it closely related to *Mycobacterium*, *Nocardia* and *Rhodococcus* (Collins & Cummins, 1986).

Among coryneform bacteria, the *Corynebacterium* plasmids are by far the best known because of the industrial potential of a number of species, which has motivated studies of their genetics. pBL1, isolated from *Corynebacterium glutamicum* (Santamaria *et al.*, 1984) has given rise to numerous cloning vectors (Martin *et al.*, 1987). Subsequently, other *C. glutamicum* plasmids, including pSR1 (Archer & Sinskey, 1993), pCG100 (Trautwetter & Blanco, 1991), pGA1 (Nesvera *et al.*, 1997) and *Corynebacterium diphtheriae* pNG2

(Serwold-Davis *et al.*, 1990) have been identified. After being widely used to develop genetic tools, pBL1, pSR1, pNG2 and pGA1 were all shown to replicate via a rolling-circle mechanism (Archer & Sinskey, 1993; Fernandez-Gonzalez *et al.*, 1994; Nesvera *et al.*, 1997; Zhang *et al.*, 1994) which might result in a serious instability behaviour, as has already been reported for pBL1 derivatives (Fernandez-Gonzalez *et al.*, 1994). The replication of the *C. glutamicum* pBL1 derivatives in *Brevibacterium linens* was reported by Sandoval *et al.* (1985) but this result could not be reproduced either in our laboratory or by Ankri *et al.* (1996). Similarly, we were unable to introduce pCG100 derivatives into *B. linens*.

To date, only three *B. linens* plasmids have been described: pBL100 (Sandoval *et al.*, 1985), a closely related plasmid pBL33 (Bigey *et al.*, 1995; Kato *et al.*, 1989) and pRBL1 (Ankri *et al.*, 1996). Nevertheless, little is known concerning their replication, except that pRBL1 is thought to replicate via a theta mechanism (Ankri *et al.*, 1996).

In this report we describe the identification and characterization of the region involved in the replication of the plasmid pBLA8, isolated from *B. linens*. We provide evidence that it belongs to a new subfamily of ColE2-

Abbreviation: HTH, helix-turn-helix.

The GenBank/EMBL accession number for the pBLA8 minimal replication region reported in this paper is Y11902.

related plasmids and describe a cloning shuttle vector derived from pBLA8 that is replicative in *Escherichia coli*, *B. linens* and *Arthrobacter*.

METHODS

Bacterial strains and plasmids. The *B. linens* strains screened for the presence of small plasmids included 26 CNRZ strains (CNRZ201–224 and 937–940) and ATCC 9172, 9174, 9175 and 19391. The strains used for pBLA8 replicative host spectrum studies were *C. glutamicum* ATCC 6872, 13058, 13287, 14020, 21488, 21649, 21792, 21086 and 21491, and the restriction-defective strains RM3 (Schafer *et al.*, 1990) and BL15 (Bonnassie *et al.*, 1990), *Arthrobacter* sp. SI55, *Arthrobacter globiformis* CCM1650, *Arthrobacter simplex* CCM1652, *Arthrobacter variabilis* CCM1565, *Arthrobacter ramosus* (Trautwetter & Blanco, 1988) and '*Arthrobacter aureus*' C70 (Le Marrec *et al.*, 1994).

Recombinant plasmids were selected in *E. coli* NM522 [*supE thi* $\Delta(lac-proAB)$ $\Delta(hsdMS-mcrB)$ 5' F' [*proAB lacIⁿ* $\Delta lacZM15$] (Mead *et al.*, 1985). The *E. coli* plasmids were: pBS8 (Spratt *et al.*, 1986), pJRD16905 (Trautwetter & Blanco, 1991), pSU20 (Bartolomé *et al.*, 1991), pBluescriptII SK+ (Stratagene) and pBK, a kanamycin-resistant homologue of pBluescriptII SK+ (Le Marrec *et al.*, 1996). These plasmids contain kanamycin- or chloramphenicol-resistance determinants, which can be selected in *E. coli* and corynebacteria but do not replicate in the latter. p21530, derived from pBL1 (Bardonnnet & Blanco, 1991), and p25450, derived from pCG100 (Trautwetter & Blanco, 1991), were used to investigate the compatibility of pBLA8 derivatives with other replicons in '*A. aureus*' C70.

Growth conditions. Strains were grown on LB medium (Miller, 1972) at 30 °C for coryneform strains and 37 °C for *E. coli*. Kanamycin, ampicillin and chloramphenicol were used at 50 $\mu\text{g ml}^{-1}$ and tetracycline at 10 $\mu\text{g ml}^{-1}$ in *E. coli* cultures. For coryneform strains, kanamycin was used at 90 $\mu\text{g ml}^{-1}$ and chloramphenicol at 3 $\mu\text{g ml}^{-1}$.

DNA manipulation. Extraction of *E. coli* plasmid DNA, and of plasmid and chromosomal DNAs from coryneform bacteria were carried out as previously described (Le Marrec *et al.*, 1994; Moreau *et al.*, 1995; Sambrook *et al.*, 1989; Trautwetter & Blanco, 1991). The DNA sequences were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical). Database searches were performed using FASTA (Pearson & Lipman, 1988) and BLAST (Altschul *et al.*, 1990) programs. Multiple sequence alignments were performed using CLUSTAL W (Thompson *et al.*, 1994).

Transformation procedures. *E. coli* cells were transformed by the method of Mandel & Higa (1970), *Arthrobacter* strains and *C. glutamicum* RM3 as described by Le Marrec *et al.* (1994), and *C. glutamicum* BL15 as reported by Bonnassie *et al.* (1990).

For transformation of *B. linens*, cells were grown to mid-exponential phase (OD_{570} 0.5) in LB medium supplemented with 10% (w/v) sodium succinate and 2% (w/v) glycine. Then 0.3 $\mu\text{g penicillin ml}^{-1}$ was added. When an OD_{570} of 1 was reached, cells were harvested, washed twice with 0.1 culture volume of cold 0.8 M sucrose, resuspended in the same solution to obtain an OD_{570} of 60 and kept on ice. For electroporation, 0.2 ml cells was mixed with plasmid DNA and immediately introduced into a pre-cooled 2 mm cuvette and pulsed with instrumental settings of 25 μF , 2.5 kV and

400 Ω . The cell suspension was immediately diluted with 0.8 ml LB medium supplemented with 0.5 M sucrose, incubated at 30 °C for 90 min and plated on LB medium supplemented with 0.5 M sucrose and the requisite antibiotic.

Isolation of a plasmid-cured derivative of *B. linens* ATCC 19391. A culture of a p18215 transformant of *B. linens* was grown overnight on LB medium supplemented with 50 $\mu\text{g kanamycin ml}^{-1}$ and 15 $\mu\text{g acridine orange ml}^{-1}$. Plasmid-free cells were identified as Kan^s clones by replica plating.

Copy number and maintenance. Cells were grown to OD_{570} 1 in LB medium containing the appropriate antibiotic. Then 40 μCi (1.48 MBq) [*methyl*-³H]thymidine was added and culture was continued for 20 min. Cells were harvested and washed twice with 0.5 ml 10 mM Tris/HCl, pH 8. Total DNA was extracted as previously described, except that proteins were extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform. An unlabelled aliquot of the plasmid of interest was added to each sample, and chromosomal and plasmid DNAs were then separated on a 0.8% agarose gel. After ethidium bromide staining, individual bands were cut out, dissolved in NaI at 50 °C and prepared for scintillation counting. The radioactivity of each band was measured, and the plasmid copy number was estimated from the total plasmid and chromosomal radioactivity, taking into account plasmid and chromosomal molecular mass (Correia *et al.*, 1994).

For determination of plasmid maintenance, plasmid-harbouring strains grown under selective conditions were diluted in fresh medium without antibiotic and grown to late exponential phase. Subcultures were then performed in the same way, without antibiotic, for 80 generations. The antibiotic-resistance phenotype of the cells of the last culture was analysed on solid LB medium in the absence or presence of kanamycin or chloramphenicol. The percentage of antibiotic-resistant clones was determined and the presence of plasmid checked on selected isolates by DNA extraction.

Compatibility with pBL1 and pCG100. '*A. aureus*' C70 harbouring p4741 (Cm^R) was electrotransformed with p21530 or p25450, Km^R derivatives of pBL1 and pCG100, respectively. The resulting transformants were selected for the incoming plasmid and then analysed by replica plating on kanamycin- and chloramphenicol-containing LB medium. The plasmid DNA content of selected colonies was analysed.

Construction of pA2209, an *E. coli*-*B. linens* shuttle vector. The *bla* gene of pBluescriptII SK+ was deleted by *Cfr*10I digestion, yielding p11915. The 3.85 kb *EcoRI*-*XhoI* fragment of p19373, containing the pBLA8 replicon and *aphIII*, was recovered. The ends were filled in with Klenow polymerase and the resulting fragment was inserted into the *DraI* site of p11915, yielding pA2209. This plasmid conserved *lacZ α* of pBluescript, facilitating the screening of recombinant plasmids in *E. coli* with *ApaI*, *Clal*, *DraI*, *HindIII*, *BamHI*, *NotI*, *SacII*, *SpeI*, *XbaI*, *EcoRI* and the unique *XhoI* site located in the multiple-cloning site. Moreover, to keep the *PstI* site in the multiple-cloning site unique, the *PstI* site present in the pBLA8 sequence was destroyed by moderate S1 digestion.

RESULTS AND DISCUSSION

Isolation and preliminary characterization of pBLA8

To obtain a plasmid able to replicate in *B. linens*, we searched for plasmids in our *B. linens* strain collection. Plasmids are not common in *B. linens* and in fact only

Fig. 1. pBLA8 derivatives and their replicative properties. The upper line shows the linearized restriction map of pBLA8; only relevant sites are indicated (their positions are given in kb). The minimal replicon is represented by a hatched box. Beneath are shown the cloned pBLA8 restriction fragments. Relevant vector restriction sites are indicated at the ends of the insert when needed to orient it within the vector, which is shown in parentheses, as are the restriction sites used for cloning. The ability (+) or inability (–) of the pBLA8 derivative to replicate in *B. linens* ATCC 19391c is indicated on the right. B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Xb, *Xba*I; X, *Xho*I.

Table 1. Features of ORFs encoded by the *Xho*I–*Pst*I region of pBLA8

ORF	Frame	Start	Stop	Shine–Dalgarno motif	Molecular mass (Da)
1	+2	17 (ATG)	280	AGGAG	8988
2	+3	668 (ATG)	985	AGGGA	11900
3 (RepA)	+2	1298 (ATG)	2248	GGACG	35562
4 (RepB)	+3	2244 (TGG)	2468	AGCGA	8559
5	+2	2467 (ATG)	2714	AGGACG	9227
6	+1	2713 (ATG)	2988	ATCCAGG	9743
7	–1	516 (ATG)	Truncated	AGTGGAGGAG	Truncated

one plasmid was found, in ATCC 19391: it was named pBLA8. Its restriction map (Fig. 1) and size of 7.5 kb, made it clearly different from *B. linens* pRBL1 (Ankri *et al.*, 1996), but similar to pBL33 (Kato *et al.*, 1989) and pBL100 (Sandoval *et al.*, 1985). This latter differs from pBLA8 by the absence of a 0.55 kb *Sal*I fragment only.

*Eco*RI-, *Hind*III-, *Pst*I- and *Bgl*II-linearized pBLA8 were inserted into the corresponding compatible sites of pBS8 and pJRD16905. The recombinant plasmids were all replicative in *B. linens* ATCC 19391, suggesting that the restriction sites used are located away from the replication functions. In every case, the resident pBLA8 was absent from the plasmid content of the transformed *B. linens* cells, facilitating the isolation of a *B. linens* ATCC 19391 plasmid-free derivative, named ATCC 19391c, by acridine orange curing. Southern blotting experiments confirmed that ATCC 19391c actually contained no DNA sequence homologous to pBLA8 (data not shown). All the constructions that were replicative in ATCC 19391 were also replicative in ATCC 19391c, so the plasmids were perfectly autonomous.

Electrotransformation of *B. linens* ATCC 19391

The electrotransformation protocol used for *C. glutamicum* strains yielded very few transformants when used for *B. linens* ATCC 19391. Nevertheless, these early transformants provided a source of homologous DNA and allowed the optimization of the

protocol. Several variables were investigated: (i) composition of the growth medium used before and following electroporation; (ii) composition of the cell-resuspension media prior to electroporation and (iii) electrical settings. The efficiency of electrotransformation obtained with the optimized procedure (described in Methods) was 5×10^4 – 1×10^5 transformants per μ g homologous DNA, but only 10–50 transformants per μ g heterologous DNA, which is prohibitive for direct cloning in *B. linens*. This protocol gave the same efficiency of transformation in the wild-type and the plasmid-cured derivative, and was used for all transformations described hereafter.

Localization of the pBLA8 minimal replicon necessary for autonomous replication

To define the minimal region of pBLA8 that is essential for its replication, various pBLA8 restriction fragments were cloned into the *E. coli* vectors pJRD16905 (Km^R) or pSU20 (Cm^R) and tested for their ability to promote plasmid replication in *B. linens* ATCC 19391c. p18136, p18215, p19373, p18399 and p4741 gave transformants in *B. linens* ATCC 19391c, whereas none was obtained with p18135 (Fig. 1). The efficiency of transformation was identical for all these plasmids, localizing the minimal replicon within the 2.5 kb *Eco*RI–*Pst*I fragment borne by p18399. When these plasmids were isolated from *B. linens* ATCC 19391c and analysed by restriction mapping, no alteration in either their size or structure

Fig. 2. Genetic organization of the *XhoI*-*PstI* fragment sustaining autonomous replication. The localization of ORFs in the *XhoI*-*PstI* fragment is indicated. pBLA8 derivatives resulted from insertion of the indicated fragment into the vector, between the restriction sites specified in parentheses. The derivatives were selected in *E. coli*. Their replication ability (+) or inability (-) was analysed in *B. linens* ATCC 19391c in the presence or absence of p4741 (Cm^R), by selection of Kan^R or $\text{Kan}^R \text{Cm}^R$ transformants, respectively. In all cases resident and incoming plasmids were present simultaneously within the cell. S, *SmaI*; N, *NcoI*; others as in Fig. 1.

was observed when compared with the corresponding plasmid purified from *E. coli*. The copy number of pBLA8 and its replicative derivatives was estimated to be 30 per chromosome equivalent, suggesting that copy number control was not affected in any derivative.

DNA sequence

The complete nucleotide sequence of the 3159 bp *XhoI*-*PstI* fragment was determined. The G + C content of this region, 62 mol%, is in agreement with the G + C content of the *B. linens* chromosome (60–64 mol%) (Jones & Keddie, 1986). The G + C content is higher than that of *C. glutamicum* plasmids pSRI (57 mol%), pBL1 (53 mol%) and pGA1 (54 mol%) (Archer & Sinskey, 1993; Filpula *et al.*, 1986; Nesvera *et al.*, 1997) and that of the *B. linens* plasmid pBBL1 (60.8 mol%) (Ankri *et al.*, 1996). Computer analysis revealed six complete ORFs oriented from left to right, and a partial one, ORF7, oriented from right to left and overlapping the 5' end of the sequenced fragment (Table 1). All these ORFs possess a putative ribosome-binding site. The start codons of ORFs 4–6 overlaps the coding region of the preceding ORF, suggesting that they are part of the same operon. Various direct and indirect repeats are located within the sequence of the minimal replicon, suggesting that they could be involved in plasmid replication.

Definition of ORFs and DNA sequences required for replication

The *EcoRI*-*PstI* minimal replicon (p18399, Fig. 1) bears neither ORF1 nor ORF7 and contains only the 3' end of ORF2. Hence, these three ORFs are dispensable for autonomous replication. This assumption was confirmed by the observation that it was possible to obtain transformants in *B. linens* ATCC 19391c with pA9394 carrying the *KpnI*-*PstI* fragment (Fig. 2). To analyse the requirement of the other ORFs for replication, we took advantage of unique restriction sites located within the region to construct deletion derivatives. ORF6 was deleted of its 3' half in pA9304 (bearing the *KpnI*-*SmaI* fragment) and was completely deleted in pA9400 (bear-

ing the *KpnI*-*EcoRV* fragment), the deletion extending into ORF5 (Fig. 2). Both plasmids replicate in ATCC 19391c; thus ORF5 and ORF6 are also dispensable for autonomous replication.

Further deletions towards the *KpnI* site abolished autonomous replication. Thus, pA7727, carrying only the 5' end of ORF4, and pB2900, deleted of ORF4 and of part of ORF3, was not replicative in ATCC 19391c. Considering the absolute requirement of ORF4 for autonomous replication, the requirement of ORF3 could not be addressed with pB2900 since, whether ORF3 is functional or not, this plasmid does not show autonomous replication. Therefore, a frameshift mutation was created within ORF3. Plasmid pA9400 was cleaved at its unique *BclI* site and the ends were filled in with Klenow polymerase prior to ligation. The resulting plasmid did not give transformants in ATCC 19391c. Thus ORF3 is also necessary for autonomous plasmid replication.

All these Km^R pBLA8 derivatives, including pB2932 (Fig. 2), gave $\text{Kan}^R \text{Cm}^R$ transformants when they were introduced into ATCC 19391c containing p4741 (Cm^R , bearing the *XhoI*-*PstI* fragment; see Fig. 1). The analysis of their restriction maps revealed that they all carried the *KpnI*-*NcoI* fragment, which does not contain any ORFs, but permits the replication of pB2932 *in trans* of p4741. The analysis of the transformant plasmid content showed that pB2932 and p4741 were both present. The *KpnI*-*NcoI* region must carry DNA signals able to promote replication in the presence of diffusible factors provided by p4741, and therefore corresponds to an *ori* site.

In summary, the minimal region required for replication could be reduced to a *KpnI*-*EcoRV* fragment that contains an *ori* site and two ORFs, ORF3 and ORF4, that have been named *repA* and *repB* respectively.

ORF7 is homologous to KfrA

Only the first 172 residues of ORF7 are encoded by the *XhoI* end of the *XhoI*-*PstI* sequence. This polypeptide is similar to the KfrA protein of plasmid RK2 (Thomas

KfrA MELNKE--TRDRIPAAADELFEGQDRENFFTVDAVRKAAAVNMNDASAGMREWRRTQAQAAPLAVQVFD 68
 ORF3 pBLA8 MTDDQSPETRA--EAAARDLADTGRAV---TARAIREAAKVRMAVASATAKAWNDAAESDDHETIPAVPE 65

KfrA AVQAGNQAVAAALWQAALANESLQAAGWDRNELEAVRGELADAFQAQARELEEAQARVTLLEQQ 138
 ORF3 pBLA8 DVTGALTAWADAYRAALAAVTPERDQLRSDVDALRVV---GLTADV-ETVEAERDTATTQADTLRKE 130

KfrA AAAAELAAARQQAALAEARTALAGAEQRAALATQRADEVERRAQELRAELDYAHQDARAFKEESRKTLD 207
 ORF3 pBLA8 LAARAKAAEKWSTEAAKHEAALSAYRDQHDKLNERTITLLLA 172

Fig. 3. Alignment of pBLA8 ORF7 with the KfrA protein of plasmid RK2. Identical amino acid residues are shown in bold type; conservative substitutions are marked with asterisks.

pBLA8 -----MSTASTETWGQMWLPLWPLAS-DDLLQGIYRTSRHNAIDVRYIEANPQSLNLLVVDIDHFDAAH 64
 pRBL1 -----MSTASTETWGQMWLPLWPLAS-DDLLQGIYRTSRHNAIDVRYIEANPQSLNLLVVDIDHFDALM 64
 pXZ10142 -----MRPSTRPEGRFEQLWPLWPLAS-DNLRDGIYRTSRDKALDKRYIETNPDAISNLLVVDIDKQDALL 66
 pAL5000 MGWPLSEISDLELVS DHTPVINRGLLPVSHVADEFEQLWPLWPLAS-DDLEGIYRQSRASALGRYIEANPALTANLLVVDVDPDAAL 91
 pMB1 MYAVAMSDEYSQPTLELSRTFEGWPLPERPLCC-DDDYSLRHRSSADALCKKHIEANPAALVNTIVVDIDANAKA 76
 pFAJ2600 -----METMTPERWEAHEWYGRYPLASNNPRIDGSPMPRSDALLKRFIQANPREYVSQIVVDIDHADAEL 66
 ColE5-099 -----MSAALQYFEENLPHRYHT-DDLAFGLRISGKGRALLAGYIQNQPHAQFWLVDVDRREGAA- 61
 pJD1 -----MSQLYTPDPLFQERIPHPKYCK-DFKEAPMLVRSYAAALKRRIYQVNPShLRFVFMFLDLDYEGAG- 63
 pTIK12 -----MAGTT AQKLDNQTDARFFQSGTALNRVLTEAPYLPRCS-DDKTATVRV-PREYAIRPYPMQVNRPGFVSWLIFDLHDH--AKA 78

bhhhh@YhDhh h

pBLA8 AACGTE-KPWPQNAVVENPANGHAHAVWALAEFVTRTEYA-RRKPLAYAAAVT@SLRRSVGDKGYSGSLITKNPHEQWEASWLTDH---LY 153
 pRBL1 RAMWNR-KAWQNAVVENPANGHAHAVWALAEFVTRTEYA-RRKPLAYAAAVT@SLRRSVGDKGYSGSLITKNPHEQWEASWLTDH---LY 153
 pXZ10142 RSMWDR-DGWRPNNAVVENPANGHAHAVWALAEFVTRTEYA-RRKPXAYAAAVT@SLRRSVGDKGYSGSLITKNPHEQWEASWLTDH---LY 155
 pAL5000 RALSARGSHPLFNAIVGNRANGHAHAVWALNAFVTRTEYA-RRKPLAYMAACA@SLRRRAVDGDRSYSGMLTKNPGHIAWETELHSD---LY 181
 pMB1 MALWEH-EGMRPNWIAENPANGHAHAGWVLTFFVPRFDLA-RLKPLKLLHAT@SLRRSCDGMGYSGMLTKNPGHIAWETELHSD---LY 165
 pFAJ2600 RAFSMHEVGLVFNMFAYSTRPGHQATFLRSFVSLSEAS-RRKPINLLARCQQLTVALSGDPHYSGPLARNPTEPMANTRWCHPT---PY 156
 ColE5-099 IDWSDR-NAPAPNITVKNPVNGHAHLLYALNIAVRTAPDA-SVKALKYAAAIERALCEKLGADVNSGLICKNPFHLEWLVMEWREE---AY 150
 pJD1 LAWEDN-NLMPAWAANRENGGAHLAYALSAPVLTAEYGGQKALRYLALEAAYKAKLRGDVGFVSLITKNPHEHPLWTLRGVPDAIRGY 156
 pTIK12 MIWEDA-GLPAPNLIVNRQSGHSHLYAIP-PVCTTETA-RSKPIAYMKTIVYGGLD-----FHSGPVAKTGPEHPLWTLRELHHAH---VY 159

pBLA8 NLDELTEHLTVSDFMPPDSWQRTKRNKPVGLGRNCTLFETVRWDVYVCPVSGSATNTPRPFDRHDLKQRSS-ICARRVTEF-----SEAL 238
 pRBL1 NLDELTEHLTVSDFMPPDSWQRTKRNKPVGLGRNCTLFETVRWDVYVARTIRKNEHPTPFDRHDLAAIVNLCQGMNSTF-----SEAL 239
 pXZ10142 TLDELRFWLEETGMPPEBWKTRRRKSPIGLGRNCAFFESARSWAYNERHFG-----DPDGLGRSIQATAQALNQSL-----F-SEPL 234
 pAL5000 TLSHIEAELGANMPPPWRQTTYKAAPTPLGRNCAFFDSVRLWAYFPALMR-----LYLPTRNVLDGLGRIYAECHARNAEFPNCNDVCPGL 267
 pMB1 DLEQLVQSLQEHGDMPPVSWKRTKARTQGLGRNCTLFDKARTLAYFVVAAD---R-SEASSEALRLVVRRTCHELNVSL-----F-PDPL 247
 pFAJ2600 ELRDLARALGRDLMPFP-TSRRVEASIDSALGRNCWFMFDTQWAYHAWTRYP-----QRTDWDDAVFAYTWGRNPNELVSHS--KGPL 236
 ColE5-099 TLDELADYLDLSASARR-----SIDKHYGMGRNCHLFEMTRKWAYFAIRQGW-----VFSQWLDAVIQVRVEMVNVSL-----PVPL 222
 pJD1 DLEYLADVLDLDFKFPYIG-R-SNVEAVGLSRNCTVFNLVSRWAKNVLAFAKQ---Q-GYTVQGWLKEVHYQCMRVNGF-----PVPM 236
 pTIK12 ELGELADYVDLAVSSPWGKG---PQFDEVSHSRHCILFEHLRDYAYSVNRRERE-----RGSFGFTFRVLEAYAHNRNSFQKLG--BMDNL 239

pBLA8 PASEIRATIRSFYKWIITRYTGWLD---SRTTSEEKSAAYHRNTGRQGLKGGIASAK--LAARPPASALWQSSAMATRERI PVMG----- 317
 pRBL1 PASEIRATIRSFYKWIITRYTGWLD---SRTTSQSKSAAYHRNTGRKGLKGGVVSQ--VR-RQ--KALERVSMLEAGKL----- 310
 pXZ10142 PVAEVDQIARSIIHRWIITKSRMWT--GPAVYEATFTTIQATROKRS-----AEHRWCTTNAERIERFIND----- 298
 pAL5000 PDEVEATIANBIHWITKSRMWAD--GIVVYEATLSARQSAISRKG-----AAAR--TAASTVARRAKSASAMEALL----- 336
 pMB1 HAREVEDIAKSIHKWIITKSRMWRD--GATANAATFIAIQSARGHKHG-----ENKYQVMKEALEW----- 306
 pFAJ2600 PDESLRTISRSVASFVWN-SEMRK--GAEQFDADFSKIQAARGHKGG--KTMTKE--RREVIATNRRKVDRAAVLALEA----- 310
 ColE5-099 SPAECRAIGKSIARYTHRNFTPETF--AQVADTHTPETIQAARGHKGGKIQGAKSKRGAVATSARTLKPWTLGISRAWYQYQKKRGLVE 308
 pJD1 WEKEVKCTSKSIANWVWYKFDIAAS--NRRFSELQ--AHNRLAKT-----TINAGRTKITEL----- 289
 pTIK12 AQSSLKGTVMKVARWTDRTYTGWRCHRGVMQLDKDPLTERASAPPPAPTGHKTTESSCAPRAACCNRRKPERKPLSAA----- 320

Fig. 4. Alignment of the pBLA8 RepA deduced amino acid sequence with homologous Rep proteins. ColE5-099 Rep is representative of the ColE2-related plasmid family. Two positions with presumptive frameshifts in pXZ10142 RepA sequence are translated as an X, as previously proposed (De Mot *et al.*, 1997). Bold characters indicate the residues of RepA from pBLA8 that are conserved in the other proteins. Positions with identical residues in all the sequences are indicated (*). The consensus motif of primases is shown above the alignment. The boxed sequence indicates the non-conserved region.

et al., 1995) (Fig. 3). KfrA is a protein of 308 residues (Thomas *et al.*, 1995) and is almost entirely α -helical (Jagura-Burdzy & Thomas, 1992). This property was also predicted for the truncated ORF7. The typical extended coiled-coil domain of KfrA, containing a heptad repeat segment, is located outside the sequenced region of ORF7. The region containing the helix-turn-helix (HTH) domain of KfrA is poorly conserved in ORF7, and such a motif was not predicted. KfrA was

reported to be involved in plasmid partitioning; nevertheless its inactivation did not significantly alter the plasmid copy number. The same observation could be made for pBLA8 ORF7, since it is destroyed in several pBLA8 derivatives that proved to be as stable as the native plasmid. Hence, if present on pBLA8, partition functions encoded by ORF7 are not crucially important for the maintenance and heredity of this high-copy-number plasmid.

pJD1	-----MAYPKLTKTKRDVTAKELAKRFQGCSTRTVFRWASQSRADYLAENSISRDKPWEHFGISRATWYRRGKMPMPSETDNQSE---TA-----	80
pAL5000	MSDGYSDGYSNGYRPTVRKKRRVTAAEGARITGLSERHVVRLVAQERSEWLAEQAAARRERIRAYHDDDEGHWPQTAKHFGHLDVTVKRLGYRARKERAAEQAAQKAHNEADNPPLF-	119
pMB1	-----MVRTTLRKKRFVSARELAAYGVSTRTIQSVVAMKREDWIDEQAAMREAVRSYHDDDEGHWTPEQTAEHFNMSQGAVRRCYRARKF-----EDEAAEKSKHLPGEIPLFD	105
pBLA8	-----MADYRELAERAGVDPRTIKRWTSPPREYLSRANSGLRIRLELRRTGLSMRTIANEVGCSVGTVHN-----ALKLQPEDESS-----	75
pXZ10142	-----MTKRTRIPRNGKTIAREVAEGTGLSTATIERWTSAPREDYLAQANEKRVVQELRAKGLSMRALAEIGCSVGLVHR-----YVKEVE-EKKT-----	87
pFAJ2600	-----MPAENQTRRNKTAKEBMAERLGVSERTIRNIVAESRDSYQARAERRDTAVKLREQ-GMKYREIAEEMEISTGAVGRLLHDAKHD-----EKNIESRAS-----	93
	HTH 1	HTH 2

Fig. 5. Sequence alignment of RepB from pBLA8. The RepB putative protein sequence was aligned with homologues from plasmids pAL5000, pMB1, pXZ10142, pFAJ2600 and pJD1. Bold characters indicate the residues of RepB from pBLA8 that are conserved in the other proteins. The HTH motifs (1 and 2), predicted by the method of Dodd & Egan (1990), are boxed.

RepA is homologous to replication proteins associated with theta replication

A search of the GenBank protein database revealed that RepA is related to Rep proteins of various plasmids. The best scores corresponded to the Rep protein of pRBL1 (from *B. linens*) (Ankri *et al.*, 1996), RepA of pAL5000 (*Mycobacterium fortuitum*) (Stolt & Stoker, 1996), RepA of pMB1 (*Bifidobacterium longum*) (Rossi *et al.*, 1996), RepA of pJD1 (*Neisseria gonorrhoeae*) (Korch *et al.*, 1985), Rep of pTiK12 (*Thiobacillus intermedius*) (English *et al.*, 1995) RepA of pXZ10142 (*C. glutamicum*) (accession no. X726910), RepA of pFAJ2600 of *Rhodococcus erythropolis* (De Mot *et al.*, 1997) and the Rep proteins of ColE2-related plasmids: ColE2-GEI602, ColE2-CA42, ColE6-CT14, ColE7-K317, ColE4-CT9, ColE5-099, ColE9-J, ColE2imm-K317, ColE3-CA38, ColE8-J and ColE2-P9 (Hiraga *et al.*, 1994). These protein sequences were aligned using the CLUSTAL W program. As the Rep proteins encoded by ColE2-related plasmids vary by a few residues only, we used ColE5-099 as the representative in the plasmid comparisons shown in Fig. 4.

We did not observe any homology with the Rep proteins of other plasmids, notably the rolling-circle-replication Rep proteins. Moreover, no DNA motif characteristic of these plasmids was identified. Since the rolling-circle-replication plasmids characterized so far are highly interrelated, as shown by Gruss & Ehrlich (1989), pBLA8 presumably does not use this mode of replication. pBLA8 does not share significant homology with *C. glutamicum* plasmids pBL1, pSR1, pNG2 or pGA1. The Rep proteins of pSR1, pNG2 and pGA1, which replicate via the rolling-circle mechanism, are related, but show no significant homology with that of pBLA8. pBLA8, pRBL1 and pXZ10142 constitute a new family of theta-replicating plasmids in corynebacteria.

RepB is homologous to other RepB proteins

RepB is required for plasmid replication. Database searches revealed significant homology with RepB proteins encoded by pJD1, pAL5000, pMB1, pXZ10142 and pFAJ2600. The most highly conserved region is located at the N terminus of these proteins. Using the method of Dodd & Egan (1990), an HTH motif (HTH 1, Fig. 5) was predicted in this region. De Mot *et al.* (1997) described a second HTH motif (HTH 2, Fig. 5) at

the C-terminus of the proteins encoded by pXZ10142 and pFAJ2600; this motif is also present in the pBLA8 RepB protein.

In pBLA8, as in pAL5000, pMB1, pXZ10142, pJD1 and pFAJ2600, *repB* is located downstream of, and overlaps, *repA*, suggesting that they are both part of the same operon. The RepB protein of pAL5000 was shown to bind to DNA near the *ori* site (Stolt & Stoker, 1996). Considering the presence of an HTH motif and the homology with pAL5000, we could speculate that the other RepB proteins have the same function. Available sequence data concerning pRBL1 cover only *repA*, but nevertheless the minimal region necessary for autonomous replication is larger than *repA* (Ankri *et al.*, 1996). So, considering the high homology with pBLA8, the existence of a *repB* gene in this region is highly probable. pTiK12 has a single Rep protein (English *et al.*, 1995). It is noteworthy that this Rep protein is also the most distant from pBLA8 RepA and is very close to ColE2 Rep.

The *ori* region

When comparing the pBLA8 region containing the *ori* site with the DNA sequence of related plasmids, the conserved motif TCAGAT was found in all the plasmids except for pFAJ2600, where only a TC--AT motif was present at the same position (Fig. 6). This motif is located upstream of *repA* and is complementary to the ppApGpA initiator RNA, found in the *ori* region of ColE2 plasmids (Hiraga *et al.*, 1994). It is positioned close to the RepB-binding site in pAL5000 (Stolt & Stoker, 1996). So, considering the homology with the ColE2 initiation site, it could be speculated that the replication fork is initiated at this site and moves unidirectionally in the opposite direction to *rep* gene transcription.

pBLA8 belongs to a Gram-positive subfamily of ColE2-related plasmids

The results of the RepA sequence alignment (Fig. 4) clearly show that all the Rep proteins analysed are homologous and several amino acids are conserved in all of them. Thus they belong to the theta replicases of the ColE2 family. The existence of additional conserved motifs among the RepA proteins of pBLA8, pRBL1, pXZ10142, pAL5000 and pMB1 suggests that these

	distance from RepA start codon	
pBLA8	-68	GAAAGCAATAT TCAGATGGTTCCGGC
pRBL1	-68	GAAAGCAATAT TCAGATGGTTCCCTGC
pMB1	-26	GGGAACCAAG TCAGATATTT CAGCC
pXZ10142	-65	CGTAACCAAG TCAGATGTTTCCGGG
pAL5000	-45	GAGCTCCAAG TCAGATATTT CGCTG
pJD1	-106	ATAACCATAAT TCAGATAACAGCCCG
pFAJ2600	-89	AGTCAGGTAG TCGAATTTT GGAGC
ColE2		ATAAGCCTTAT TCAGATAACAGCGCC *** →

Fig. 6. Alignment of the putative *ori* site of pBLA8, pRBL1, pMB1, pXZ10142, pFAJ2600, pIL5000 and pJD1 with the ColE2 *ori* site. Except for ColE2, all the *ori* sequences analysed are located upstream of the *rep* operon and they work in the opposite direction to *rep* transcription. The distance of their 5' end from the Rep translation initiation codon is indicated. Bold characters indicate pBLA8 nucleotides that are highly conserved in the other sequences. The primer RNA (5'ppApGpA) for leading strand synthesis (arrow) for ColE2 plasmids is indicated (***).

proteins may constitute a Gram-positive Rep subfamily of ColE2-related plasmids. This family would be characterized by proteins containing two conserved regions spaced by a poorly related region, in contrast to the Rep proteins of other ColE2-related plasmids, which are highly homologous throughout their sequences except in the last 30 residues (Hiraga *et al.*, 1994). The N-terminal region is the most highly conserved domain and, furthermore, it contains most of the motifs shared with the Rep proteins of ColE2-related plasmids. This region is involved in primase activity (Hiraga *et al.*, 1994). Moreover, the amino acid sequence motif described for bacterial primases (Pansegrau & Lanka, 1992) is present in all members of the pBLA8 RepA subfamily. The spacer region is weakly conserved in pBLA8 and pRBL1 Rep proteins, not at all in the Rep proteins originating from other Gram-positive bacteria, and is clearly divergent from the Rep proteins of ColE2-related plasmids. The C-terminal domain is poorly homologous among these proteins, except for pBLA8 and pRBL1, which share significant homology. It is also the most divergent region in the Rep proteins of ColE2-related plasmids. It contains the HTH motif interacting with *ori* and responsible for *ori* binding specificity (Hiraga *et al.*, 1994). Such a motif is lacking in RepA proteins of the pBLA8 subfamily.

The existence of this subfamily was confirmed at the DNA level. A database search for DNA homologies among pBLA8 and DNA sequences, revealed strong homology throughout the sequence with pRBL1 (84%) but restricted to the sequence encoding the N-terminal domain of the Rep protein from pXZ10142 (72%), pAL5000 (67%) and pMB1 (57%). This suggests an ancestral replication module common to all these plasmids that emerged after, or independently of, the highly conserved module in the ColE2 family. This REP module, common to all the proteins analysed, encoded

primase activity. Each Rep DNA-binding site and *ori* site evolved simultaneously in each plasmid. Such a mosaic structure, and evolution by the exchange of functional parts of the replicon via homologous recombination, has been described for ColE2 derivatives (Hiraga *et al.*, 1994). These replication functions originate from a common ancestor which invaded the eubacteria of different phyla and evolved divergently to optimize host/plasmid interactions.

The ColE2 Rep protein has two activities. First, it binds specifically to *ori* (Shinohara & Itoh, 1996). Second, the protein is a site-specific primase, since it is able to synthesize a short RNA molecule from a supercoiled template (Takechi *et al.*, 1995). In pBLA8-related plasmids, RepB proteins (DNA-binding) should act together with RepA (primase) to initiate DNA replication in a way similar to Rep of ColE2, which possesses both activities. In all these plasmids, RepA and RepB seem to be encoded by a single operon, *repAB*. Another structural feature of these plasmids is the location of the *ori* site upstream of *repAB*, the replication fork moving in the opposite direction to *repAB* transcription. *ori* is located downstream of the *rep* gene in ColE2, the replication fork moving in the same direction as *rep* transcription. Considering the nucleotide sequence of the *ori* site (Fig. 6), we can distinguish two groups, the first including pRBL1, pBLA8, pMB1, pXZ10142 and pAL5000, the second including pJD1 and the ColE2 family. The *ori* site of pFAJ2600 is unique. This grouping is identical to that observed for RepA proteins and must have a functional significance. Thus, considering all these structural differences, it is clear that the ColE2 family is composed of two structural subfamilies, the first including ColE2 derivatives and probably pTIK12, and the second made up of pBLA8, pRBL1, pXZ10142, pAL5000, pMB1, pFAJ2600 and pJD1.

pA2209: an *E. coli*-*B. linens* shuttle vector

To construct an efficient shuttle vector, we used pBluescriptII SK+, considering its advantages for the direct selection of recombinant plasmids in *E. coli*. It was modified by adding pBLA8 replication functions and a phenotypic marker active in corynebacteria (Fig. 7).

The stability of pA2209 was analysed in ATCC 19391c. After 80 generations without antibiotic, only 2% of the cells had lost the plasmid. Hence it could be used for continuous-culture fermentation in the absence of any selective pressure. This stability was not modified by introducing an *EcoRI* fragment bearing a chloramphenicol-resistance determinant into the vector (data not shown).

Host spectrum of pBLA8 derivatives

When pA2209 was introduced by electroporation into other corynebacteria, no Kan^R colonies were recovered with any of the *C. glutamicum* strains analysed, in-

Fig. 7. *E. coli*-*B. linens* shuttle vector pA2209. *, Sites destroyed following Klenow polymerization; ●, sites destroyed by S1 limited digestion. MCS (multiple-cloning site) contains (clockwise) KpnI, ApaI, DraI, XhoI, HincII, SalI, ClaI, HindIII, EcoRV, EcoRI, PstI, SmaI, BamHI, SpeI, XbaI, NotI, SacI, SacI. Bold restriction sites indicate unique sites.

cluding BLI5 and RM3, in spite of the fact that these restriction-defective strains were efficiently transformed with p21530 and p25450, the derivatives of pBL1 and pCG100 respectively. In contrast, Kan^R colonies were obtained with *Arthrobacter* sp. SI55, '*A. aureus*' C70, *A. globiformis* and *A. ramosus* with a high efficiency (10^6 transformants per μ g DNA), identical to that obtained when p21530 and p25450 were transformed in *C. glutamicum* BLI5 and RM3. '*A. aureus*' C70 had been previously shown to have no restriction activity against *E. coli* DNA (Le Marrec *et al.*, 1994). *Arthrobacter* sp. SI55 and *A. globiformis* show the same behaviour. The host spectrum did not include all *Arthrobacter* strains, since *A. simplex* and *A. variabilis* could not be transformed by pBLA8 derivatives. This discrepancy in the *Arthrobacter* strain behaviour should perhaps be considered in the context of the heterogeneity of this genus. Thus, the cell wall of *A. variabilis*, containing meso-diaminopimelate and mycolic acids, indicates that it is closely related to the genus *Corynebacterium* (Keddie *et al.*, 1986). Likewise *A. simplex* is also phenotypically and genomically distinct from *Arthrobacter sensu stricto*, and it has been proposed that this bacterium should also be reclassified (Keddie *et al.*, 1986).

The stability of pA2209 was assayed in these strains. In each case, only 3% of the cells lost the plasmid after 80 generations in the absence of selective pressure. In spite of the fact that pBLA8 and pRBL1 are highly homologous and originate from *B. linens*, they have different host spectra. Thus only pRBL1 derivatives are able to replicate in *C. glutamicum* (Ankri *et al.*, 1996). pBL33

and pBL100 have a restriction map identical to that of pBLA8, yet pBL33 was shown to replicate in *Rhodococcus* sp. R312 (formerly *Brevibacterium* sp. R312) (Bigey *et al.*, 1995). Since other bacteria have not yet been tested, it is difficult to compare the host spectrum of pA2209 with that of pBL33.

Compatibility of the pBLA8 derivative p4741 (Cm^R) with p21530 (pBL1 replicon, Km^R) and p25450 (pCG100 replicon, Km^R) was investigated in '*A. aureus*'. Both pairs of plasmids were maintained in the presence of kanamycin and chloramphenicol. In the absence of selective pressure, p25450 and p4741 were both found to be present after 80 generations. Because of the intrinsically high instability of p21530 (it is lost after 10 generations), the strain containing p21530 and p4741 contains only p4741 after 10 generations in the absence of antibiotic. In the presence of kanamycin, both plasmids were present after 80 generations. Thus pBLA8 is compatible with pBL1 and pCG100. The compatibility of pBL1 derivatives and pBL100 has also been described by Sandoval *et al.* (1985).

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REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Ankri, S., Bouvier, I., Reyes, O., Predali, F. & Leblond, G. (1996). A *Brevibacterium linens* pRBL1 replicon functional in *Corynebacterium glutamicum*. *Plasmid* **36**, 36–41.
- Archer, J. A. C. & Sinskey, A. J. (1993). The DNA sequence and minimal replicon of the *Corynebacterium glutamicum* plasmid pSR1: evidence of a common ancestry with plasmids from *C. diphtheriae*. *J Gen Microbiol* **139**, 1753–1759.
- Bardonnet, N. & Blanco, C. (1991). Improved vectors for transcriptional signal screening in corynebacteria. *FEMS Microbiol Lett* **84**, 97–102.
- Bartolomé, B., Jubete, Y., Martinez, E. & de la Cruz, F. (1991). Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. *Gene* **102**, 75–78.
- Bigey, F., Grossiord, B., Chan Kwo Chion, C. K., Arnaud, A. & Galzy, P. (1995). *Brevibacterium linens* pBL33 and *Rhodococcus rhodochrous* pRC1 cryptic plasmids replicate in *Rhodococcus* sp. R312 (formerly *Brevibacterium* sp. R312). *Gene* **154**, 77–79.
- Bonnassie, S., Oreglia, J., Trautwetter, A. & Sicard, A. M. (1990). Isolation and characterization of a restriction and modification deficient mutant of *Brevibacterium lactofermentum*. *FEMS Microbiol Lett* **72**, 143–146.
- Collins, M. D. & Cummins, S. C. (1986). Genus *Corynebacterium*. In *Bergey's Manual of Systematic Bacteriology*, p. 1266. Edited by J. G. Holt. Baltimore: Williams & Wilkins.
- Correia, A., Martin, J. F. & Castro, M. (1994). Pulsed-field gel

electrophoresis analysis of the genome of amino acid producing corynebacteria: chromosome sizes and diversity of restriction patterns. *Microbiology* 140, 2841–2847.

De Mot, R., Nagy, I., De Schrijver, A., Pattanapitpaisa, P., Schoofs, G. & Vanderleyden, J. (1997). Structural analysis of the 6 kb cryptic plasmid pFAJ2600 from *Rhodococcus erythropolis* NI86/21 and construction of *Escherichia coli*–*Rhodococcus* shuttle vectors. *Microbiology* 143, 3137–3147.

Dodd, I. B. & Egan, J. B. (1990). Improved detection of helix-turn-helix DNA-binding motifs in protein sequences. *Nucleic Acids Res* 18, 5019–5026.

English, R. S., Lorbach, S. C., Huffman, K. M. & Shively, J. M. (1995). Isolation and characterization of the replicon of a *Thiobacillus intermedius* plasmid. *Plasmid* 33, 1–6.

Fernandez-Gonzalez, C., Cadenas, R. F., Noirot-Gros, M. F., Martin, J. F. & Gil, J. A. (1994). Characterization of a region of plasmid pBL1 of *Brevibacterium lactofermentum* involved in replication via the rolling circle model. *J Bacteriol* 176, 3154–3161.

Filpula, D., Ally, A. H. & Nagle, J. (1986). Complete nucleotide sequence of a native plasmid of *Brevibacterium lactofermentum*. *Nucleic Acids Res* 14, 5114.

Gruss, A. & Ehrlich, S. D. (1989). The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. *Microbiol Rev* 53, 231–241.

Hiraga, S.-I., Sugiyama, T. & Itoh, T. (1994). Comparative analysis of the replicon regions of eleven ColE2-related plasmids. *J Bacteriol* 176, 7233–7243.

Jagura-Burdzy, G. & Thomas, C. M. (1992). *kfrA* gene of broad host range plasmid RK2 encodes a novel DNA-binding protein. *J Mol Biol* 225, 651–660.

Jones, D. & Keddle, R. M. (1986). Genus *Brevibacterium*. In *Bergey's Manual of Systematic Bacteriology*, p. 1301. Edited by J. G. Holt. Baltimore: Williams & Wilkins.

Kato, F., Hara, N., Matsuyama, K., Hattori, K., Ishii, M. & Murata, A. (1989). Isolation of plasmids from *Brevibacterium*. *Agric Biol Chem* 53, 879–881.

Keddle, R. M., Collins, M. D. & Jones, D. (1986). Genus *Arthrobacter*. In *Bergey's Manual of Systematic Bacteriology*, p. 1288. Edited by J. G. Holt. Baltimore: Williams & Wilkins.

Korch, C., Hagblom, P., Ohman, H. M. G. & Normark, S. (1985). Cryptic plasmid of *Neisseria gonorrhoeae*: complete nucleotide sequence and genetic organization. *J Bacteriol* 163, 430–438.

Le Marrec, C., Michotey, V., Blanco, C. & Trautwetter, A. (1994). ϕ AAU2, a temperate bacteriophage specific for '*Arthrobacter aureus*', whose integrative functions work in other corynebacteria. *Microbiology* 140, 3071–3077.

Le Marrec, C., Moreau, S., Loury, S., Blanco, C. & Trautwetter, A. (1996). Genetic characterization of site-specific functions of ϕ AAU2 infecting '*Arthrobacter aureus*' C70. *J Bacteriol* 178, 1996–2004.

Mandel, M. & Higa, A. (1970). Calcium-dependent bacteriophage DNA infection. *J Mol Biol* 53, 159–162.

Martin, J. F., Santamaria, R., Sandoval, H., Del Real, G., Mateos, L. M., Gil, J. A. & Aguilar, A. (1987). Cloning systems in amino acid-producing corynebacteria. *Bio/Technology* 5, 137–146.

Mead, D. A., Skorupa, E. S. & Kemper, B. (1985). Single stranded DNA SP6 promoter plasmid for engineering mutant RNAs and proteins: synthesis of a 'stretched' preproparathyroid hormone. *Nucleic Acids Res* 13, 1103–1118.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor: Cold Spring Harbor Laboratory.

Moreau, S., Leret, V., Le Marrec, C., Varangot, H., Ayache, M., Bonnassie, S., Blanco, C. & Trautwetter, A. (1995). Prophage distribution in coryneform bacteria. *Res Microbiol* 146, 493–505.

Nesvera, J., Patek, M., Hochmannova, J., Ahrhmová, Z., Becvarova, V., Jelinkova, M. & Vohradsky, J. (1997). Plasmid pGA1 from *Corynebacterium glutamicum* codes for a gene product that positively influences plasmid copy number. *J Bacteriol* 179, 1525–1532.

Pansegreau, W. & Lanka, E. (1992). A common sequence motif among prokaryotic DNA primases. *Nucleic Acids Res* 20, 4931.

Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* 85, 2444–2448.

Rossi, M., Brigidi, P., Gonzalez Vara y Rodriguez, A. & Matteuzzi, D. (1996). Characterization of the plasmid pMB1 from *Bifidobacterium longum* and its use for shuttle vector construction. *Res Microbiol* 147, 133–143.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory.

Sandoval, H., Del Real, G., Mateos, L. M., Aguilar, A. & Martin, J. F. (1985). Screening of plasmids in non-pathogenic corynebacteria. *FEMS Microbiol Lett* 27, 93–98.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74, 5463–5467.

Santamaria, R., Gil, J. A., Mesas, J. M. & Martin, J. F. (1984). Characterization of an endogenous plasmid and development of cloning vectors and a transformation system in *Brevibacterium lactofermentum*. *J Gen Microbiol* 130, 2237–2246.

Schafer, A., Kalinowski, J., Simon, R., Seep-Feldhaus, A.-H. & Puhler, A. (1990). High-frequency conjugal plasmid transfer from gram-negative *Escherichia coli* to various gram-positive coryneform bacteria. *J Bacteriol* 172, 1663–1666.

Serwold-Davis, T. M., Groman, N. B. & Kao, C. C. (1990). Localization of an origin of replication in *Corynebacterium diphtheriae* broad host range plasmid pNG2 that also functions in *Escherichia coli*. *FEMS Microbiol Lett* 66, 119–124.

Shinohara, M. & Itoh, T. (1996). Specific determinants in interaction of the initiator (Rep) proteins with the origins in plasmids ColE2-P9 and ColE3-CA38 identified by chimera analysis. *J Mol Biol* 257, 290–300.

Spratt, B. G., Hedge, P. J., Te Heesen, S., Edelman, A. & Broome-Smith, J. K. (1986). Kanamycin-resistant vectors that are analogues of plasmids pUC8, pUC9, pEMBL8 and pEMBL9. *Gene* 41, 337–342.

Stolt, P. & Stoker, N. G. (1996). Functional definition of regions necessary for replication and incompatibility in the *Mycobacterium fortuitum* plasmid pAL5000. *Microbiology* 142, 2795–2802.

Takechi, S., Matsui, H. & Itoh, T. (1995). Primer RNA synthesis by plasmid-specified Rep protein for initiation of ColE2 DNA replication. *EMBO J* 14, 5141–5147.

Thomas, C. M., Smith, C. A., Ibbotson, J. P., Johnston, L. & Wang, N. (1995). Evolution of the *korA*–*oriV* segment of promiscuous IncP plasmids. *Microbiology* 141, 1201–1210.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22, 4673–4680.

Trautwetter, A. & Blanco, C. (1988). Isolation and preliminary

characterization of twenty bacteriophages infecting either *Brevibacterium* or *Arthrobacter* strains. *Appl Environ Microbiol* **54**, 1466–1471.

Trautwetter, A. & Blanco, C. (1991). Structural organization of the *Corynebacterium glutamicum* plasmid pCG100. *J Gen Microbiol* **137**, 2093–2101.

Zhang, Y., Praszler, J., Hodgson, A. & Pittard, A. J. (1994). Molecular analysis and characterization of a broad-host-range plasmid, pEP2. *J Bacteriol* **176**, 5718–5728.

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