

ARTICULOS ORIGINALES COMPLETOS/ FULL ORIGINAL PAPERS

CHARACTERIZATION OF PHAGE 2C AUTOREPLICATIVE SEQUENCES IN A STABLE *B. subtilis* PLASMID

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SUMMARY

Genetic engineering in *B. subtilis* has been hindered by the segregational instability of the plasmids commonly used, which originate from *Staphylococci*.

In search of a replicon, endogenous to *B. subtilis*, we decided to clone autoreplicative sequences of the lytic phage 2C of *B.subtilis*. For that purpose, a pUC19 derivative, replicating only in *E. coli*, was constructed carrying the *Cm^r* gene of pC194 able of being expressed in *B.subtilis*. Random restriction fragments of phage 2C were inserted in this replicon-probe vector and transformed in *B.subtilis*, yielding the recombinant plasmid pCMA.

This plasmid pCMA displayed complete segregational stability in the Gram positive host, justifying further characterization of the cloned sequences. The pUC construction had suffered important rearrangements upon transformation of *B.subtilis*, as revealed by the inability of pCMA to replicate in *E.coli* and by the loss of the pUC sequences.

Southern-blot hybridizations revealed the presence of the phage sequences. These cloned viral sequences revealed homologies with the proximal part of the tetracycline-resistance gene of pBR322, one *dnaA* box, and also homologies with the ORI (+) of ϕ X174 and with some plasmids replicating by a rolling circle mechanism. We also found the (-) origin, known as *palA* sequence, of pC194 contiguous to the *CAT* gene inserted in the replicon-probe plasmid.

RESUMEN

Los trabajos de ingeniería genética en *B.subtilis* se han visto obstaculizados principalmente por la inestabilidad segregacional de los vectores comúnmente usados.

En busca de un replicón específico para *B.subtilis*, decidimos clonar secuencias autoreplicativas del fago 2C, virus lítico de *B.subtilis*. Para este propósito se construyó un derivado del pUC19 replicable sólo en *E. coli*, insertándole el gen de resistencia al cloranfenicol (*Cm^r*) del plásmido pC194 expresable en *B.subtilis*. En este derivado se insertaron a random fragmentos del fago 2C obtenidos por restricción y posteriormente se transformó en *B. subtilis* obteniéndose el plásmido pCMA, el que

resultó ser completamente estable en esta cepa, lo que justificó la profundización en el estudio y la caracterización de esta secuencia viral clonada. Se observó que esta construcción, basada en un derivado del pUC, sufrió cambios estructurales después de ser transformada en *B.subtilis*, lo que se evidenció por la incapacidad del plásmido obtenido pCMA de replicarse en *E. coli* y por la pérdida de algunas secuencias del pUC original.

Las hibridaciones realizadas usando la técnica de Southern-blot revelaron la presencia de secuencias virales en el plásmido pCMA. Estas secuencias virales clonadas revelaron homologías con secuencias del gen que codifica para la resistencia a la tetraciclina del pBR322, también con un *dnaA* box y orígenes de replicación del ϕ X174 y algunos plásmidos que se replican mediante *rolling circle mechanism*. También se encontraron secuencias del ori (-) conocido como *palA* del pC194 a continuación del gen *CAT* insertado en nuestro replicón.

INTRODUCTION

The Gram-positive bacteria *B.subtilis*, are non-pathogenic organisms that have natural secretory systems and are widely used in industrial fermentations. For these reasons, they offer considerable advantages for the production of recombinant proteins.

Yet, their use has been limited by the segregational and structural instability of recombinant plasmids after transformation of the host (1). Most of the vectors used up to now originate from *Staphylococci*, replicating by a rolling circle mechanism. This mechanism involves the production of single-stranded DNA intermediates, which are supposed to entail their instability (2).

Hence, we turned to a replicon, endogenous to *B.subtilis* and succeeded in cloning autoreplicative sequences of the lytic phage 2C of *B.subtilis* (3, 4).

For that purpose, a pUC19 derivative replicating only in *E. coli* was constructed carrying the *Cm^r* gene of pC194, able of being expressed in *B.subtilis*. Random restriction fragments of phage 2C were inserted in this replicon probe vector and transformed in *B.subtilis*, yielding the recombinant plasmid pCMA. This plasmid displayed complete segregational stability in the Gram-positive host, justifying further characterization and study of its structure and replication mechanism in view of developing novel cloning vectors in this Gram-positive host.

MATERIAL AND METHODS

Bacterial strains: *B.subtilis* SB202 (*trp*, *tyrA1* *aroB2* *hisH2*) grew in LB-broth containing tryptone (10 g/L), yeast extract (5 g/L) and NaCl (5 g/L), using pH 9. LB-broth agar for plates was prepared as above, but adding 10 g/L of agar. *E. coli* TG1 (K12, A *lac-proAB*, *supE*, *thi*, *hsdDS/F*, *traD36*, *proAB*, *lacI^qZ* AM15) grew also in LB medium. Transformation of *B.subtilis* and *E. coli* competent cells followed the current procedures (5, 6). *B.subtilis* transformant were selected by using 0.003-0.005 g/L cloramphenicol (Cm) and *E. coli* transformants by using 0.02-0.1 g/L of ampicillin (Amp).

DNAs: Vectors for cloning and sub-cloning for sequencing were pUC19 (7) and pBS (8). Primers were designed and automatically synthesized by current methods. Methods for purification of DNA fragments and preparation of DNA probes have been described by Sambrook *et al.* (9).

M. Biology techniques: Southern-blot hybridizations were performed by preparing the ³²P labelled probes by random primer using the procedure described by Southern (10) and Sambrook *et al.* (9). Nucleotide sequencing was carried out by the method of Sanger (11), using the Sequenase Kit (USB). Restriction and modification enzymes were commercial preparations obtained from Boehringer (Mannheim, West Germany) and were used according to the supplier's instructions. Plasmid segregational stability was determined according to the method described by Swinfield *et al.* (12).

RESULTS AND DISCUSSION

We attempted to insert some phage autoreplicative regions in an *E.coli* plasmid. The replicon-probe plasmid pCM3 (figure 1) was constructed inserting the *CAT* gene of plasmid pC194, able of being expressed in *B.subtilis* in the *Amp^r* gene of pUC19 which replicates only in *E.coli*. For this purpose, phage 2C DNA (150 000 bp) was submitted to partial Sau3AI hydrolisis, mixed with pCM3 linearized by BamHI, ligated and transformed into *B.subtilis*, yielding the recombinant plasmid pCMA. This recombinant plasmid (pCMA) is the outcome of important rearrangements suffered by the transforming hybrid pCM3 carrying phage sequences. We proved that:

- 1) pCMA has lost the ability to replicate in *E. coli*.
- 2) The polylinker site has been lost and the number of other restriction sites in pCMA was lower than

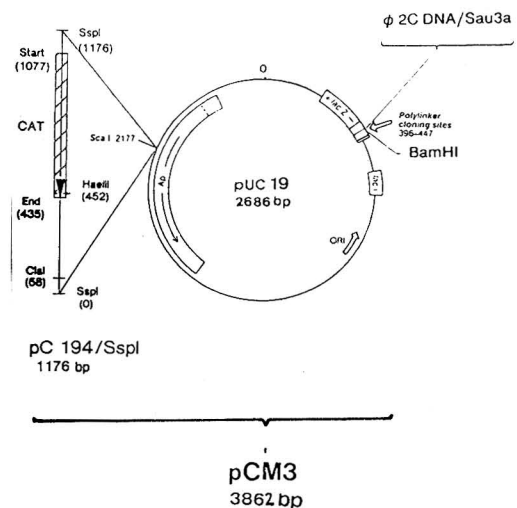


Fig. 1 Replicon-probe plasmid pCM3 and cloning of phage 2C sequences.

their number in pUC19 or pCM3. The restriction map of pCMA (figure 2) was obtained by single and double digestions with chosen enzymes and Southern-blot hybridization using selected probes. Southern-blot also revealed that the phage sequences were essentially carried by the 1050 bp fragment delineated by two HaeIII cutting sites as shown in figure 2. In order to investigate if this phage sequences were really able to drive plasmid replication, pCMA was submitted to partial hydrolisis by Sau3AI, T4-DNA ligase, transformation in *B.subtilis* and selection in a Cm containing medium. One recombinant containing a plasmid shorter than pCMA was obtained; its physical map was established and is shown in figure 3. We can observe that almost all of the viral sequence

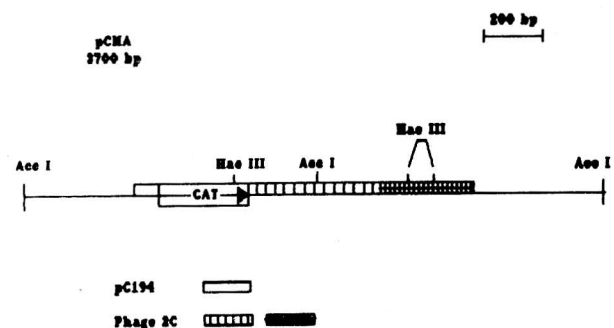


Fig. 2. Physical map of plasmid pCMA. Phage sequences are indicated by shaded boxes. The right part are sequences identical to pBR322 sequences.

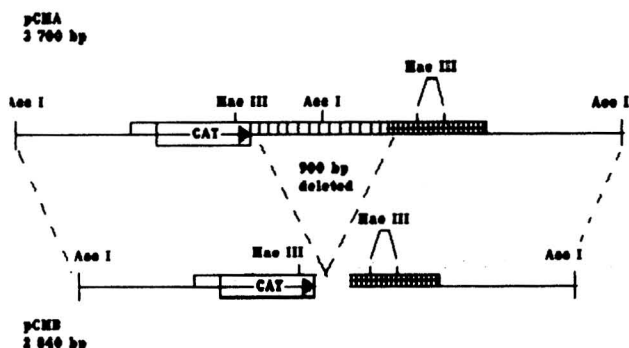


Fig.3. Deletion derivative pCMB; its comparison with pCMA. Phage sequences are hatched.

had been deleted from this recombinant. This new shorter plasmid was named pCMA. On the other hand, pCMA was hydrolyzed also with HaeIII, yielding three fragments (2540 bp, 1000 bp and 123 bp long) that were electroeluted from the agarose gel and separately inserted into pCM3 (pUC19 carrying the *CAT* gene) and transformed into *E. coli*. The recombinant obtained from the smaller and medium HaeIII fragment of pCMA did not yield recombinants upon transformation of *B. subtilis*, so we conclude that the phage sequences contained in these fragments of pCMA are not able to drive replication by themselves in this host. Upon transformation of the host with pCM3 carrying the large HaeIII fragment (2540 bp) called pCMH, recombinants were readily obtained. The pCMH physical map (not shown) indicated that it had not suffered any rearrangements upon transformation of *B. subtilis*. Segregational stability determination was carried out to these 3 plasmids. After overnight culture in the presence of chloramphenicol, recombinants pCMA, pCMB and pCMH were transferred to antibiotic-free LB broth, samples were spread on LB petri dishes and isolated colonies were tooth-picked onto a Cm-containing medium. Results (figure 4) show the perfect stability of pCMA, whereas pCMB, deleted of most or all of the phage sequences, shows a progressive loss of the plasmid. We conclude that the phage sequences might thus contain stability functions pCMH on the other hand, is very unstable. The copy number of these plasmids was determined and it

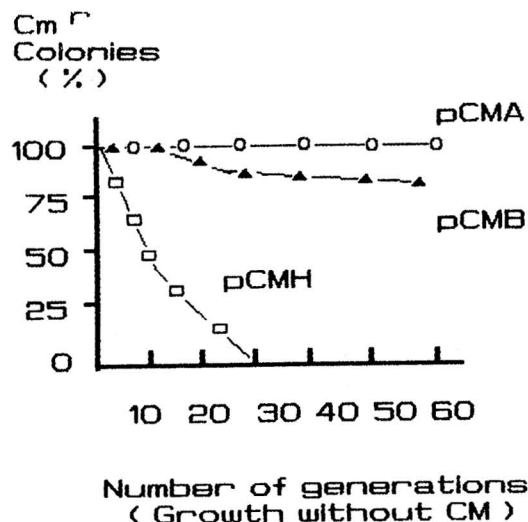


Fig. 4. Segregation stability of plasmids pCMA, pCMB and pCMH.

was very similar in all three cases, about 25 to 30 copies per cell. Another of our purposes was the localization of the cloned viral sequences in the genome of phage 2C. The available restriction map of this phage is shown in figure 5 (left). When labeled pCMA was hybridized to phage 2C, restricted with SalI, HaeIII + SalI or BglII, multiple hybridization signals appeared as shown in figure 5 (right), in which we can see three fragments in the case of SalI and HaeIII+ SalI, and five in the case of BglII (we can see in this case that the heaviest fragments in each case are not separated). Hybridization to an even number

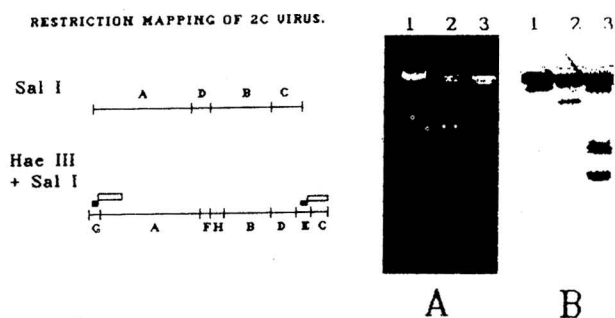


Fig. 5. Localization of cloned sequences in the phage 2C genome. Left: White boxes indicate the left side and black boxes the right side of the redundant ends. Right: Lane 1: 2C DNA, cut by Sal I. Lane 2: 2C DNA, cut by HaeIII + SalI. Lane 3: 2C DNA, cut by Bgl II. A: Visualization of the gel under UV (White dots indicate HaeIII + SalI fragment E). B: Hybridization with a pCMA probe (by ECL).

to an even number of fragments suggests that they belong to the terminal redundancies previously described (3); we can also say that hybridization to an uneven number of fragments suggests that, in addition, pCMA contains unique sequences of the 2C genome. The cloned sequences seem to originate from the right-half of the terminal redundancies. This is suggested by the absence of hybridization of pCMA with the *Hae*III + *Sal*I E fragment of phage 2C, known to contain the left-half of the terminal redundancy (3), indicated by white dots in figure 5A (Right).

Plasmid pCMA was used as a probe in hybridization with *Sau*3AI restricted 2C DNA, yielding a large number of fragments (figure 6, lane 1). This revealed four viral restriction fragments amounting to about 1200 bp. This plasmid was also restricted with *Sau*3AI (figure 6, lane 5). Hybridization to a specific 2C restriction fragment (*Bgl*III-J) revealed one viral sequence of about 150 bp, corresponding to one specific fragment of the viral genome (lane 4), also revealed by pCMA (lane 3 and 5). In these experiments, restriction fragments of pBR322 (*Hinf*I + *Bgl*III mixtures of separate digests) were used as molecular weight markers (figure 6 Line 2). pCMA, used as a probe, revealed consistently cross hybridization with pBR322 sequences (figure 6, lane 6). To evidence this cross hybridization, the three fragments making up pCMA were electrocluted from an agarose gel. Their hybridization to a *Hae*III restricted pBR322, revealed three fragments of 192, 123 and 104 bp respectively (figure 7-above, lanes 2, 3 and 4). The corresponding fragments in the known physical map of pBR322 and pBR328 is shown in



Fig.6. Southern blot hybridization. Lane 1: 2C DNA cut by *Sau*3AI (seen under UV). Lane 2: pBR322 DNA, mixture of *Hinf*I and *Bgl*II hydrolisates (seen under UV). Lane 3: same as lane 1, hybridized with a probe of pCMA. Lane 4: same as lane 1, hybridized with fragment *Bgl*III-C of 2C. Lane 5: pCMA, cut by *Sau*3AI, hybridized with fragment *Bgl*III-C of 2C. Lane 6: Restriction fragments of pBR328 (same as in lane 2) hybridized with a pCMA probe.

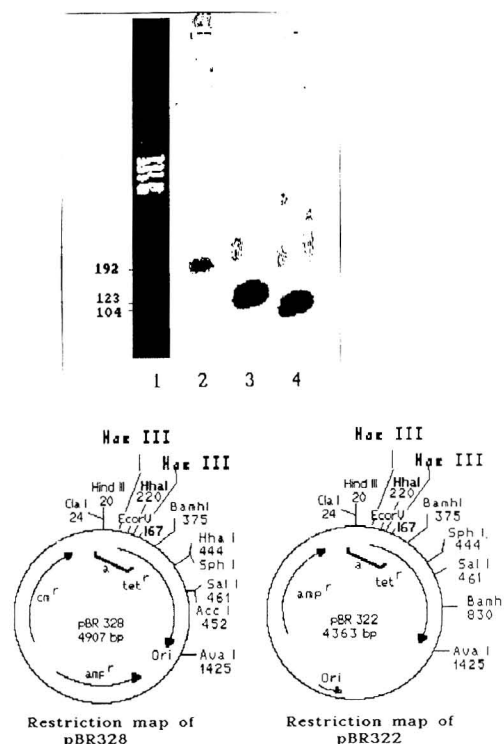


Fig.7. Hybridization between pCMA and pBR322 sequences. **Above:** Lane 1: pBR322 cut by *Hae*III (seen under UV). Lane 2 to 4: same as in lane 1, hybridized with the three isolated *Hae*III fragments of pCMA (A, B and C respectively of 2500, 1000 and 123 bp). **Below:** Physical maps of pBR322 and pBR328. The *a* indicates identical sequences found in pCMA.

figure 7 (below). They correspond to the N-terminal part of the TetA protein.

We also used pBR322 as a probe to see if the 2C DNA contained sequences homologous to this *E.coli* plasmid. This experiment (results not shown) revealed homology with sequences contained in 2C DNA restricted by several enzymes. In addition, in the same experiment, *Hae*III-restricted pBR322 showed cross hybridization with a specific 2C restriction fragment

Table 1
Southern-blot hybridization of pCMA with chosen DNAs.

DNA Probe:	Hybridization with pCMA
2C DNA	+ ⁽¹⁾
pCM3	+
pUC19	-
M13mp19	-
pBR322	+
pBR328	+
<i>B.subtilis</i> chromosomal DNA	-
Lambda DNA	-

⁽¹⁾ Identical results were obtained using pCMA as probe in hybridizations where the listed DNAs were anchored on a membrane.

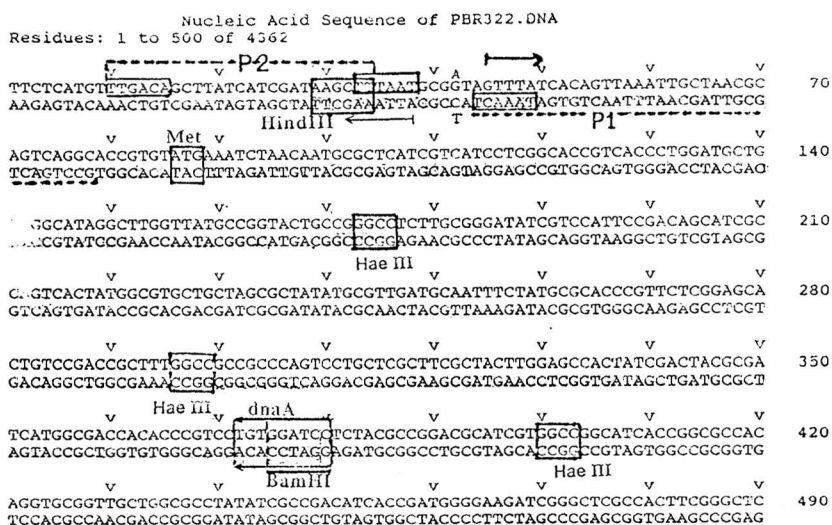


Fig. 8. Identities between pBR322 (N-terminal region of *tet* gene) and pCMA/HaeIII restriction fragments. P1 and P2: Promoters. (1) Identical results were obtained using pCMA as a probe in hybridizations where the listed DNAs were anchored on a membrane.

(BgIII-C), and no cross-hybridization of pCMA was observed with the pUC19 plasmid used in the construction of the replicon-probe plasmid

At this stage (table I) we conclude that indeed the pBR322-like sequences originated from 2C DNA.

Sequencing of pCMA confirmed identity with the pBR322 sequence identified as *a* in figure 7 (below). This fragment includes the promoter and more than the first 98 codons of the *tet* gene (figure 8) shown to be important in pBR322 for its insertion into the membrane (13). Membrane insertion of the growing TetA protein, still linked to the transcription complex via coupled transcription and translation, firmly anchors the transcription complex to a large cellular structure. This forces the DNA to twist during transcription, generating supercoils (13). This membrane-anchoring could also play a role in the stability of the plasmid. We also found homologies with a *dnaA* box contained at the end of the pBR322-like sequence (figure 8) and also homologies with (+) origin of ϕ X174 *E. coli* phage and Gram-positive plasmids pUB110, pBAA1 and pC194 (figure 9). In the other hand, we also found identity with the (-) origin of pC194 called *palA*, inserted with the *Cm^r* gene of this plasmid into pUC19 (figure 9).

In view of all the similarities found with phage 2C sequences in plasmid pCMA with the origin of replication of plasmids and phages replicating via a rolling circle mechanism involving single-stranded intermediates; we conclude that this pCMA could have this kind of rolling circle mechanism for its

replication. In order to investigate the presence of single-stranded pCMA intermediates, a total lysate of *B. subtilis* carrying pCMA was submitted to agarose gel electrophoresis in duplicate, one of them being blotted on nylon membrane without prior denaturation, thus retaining single-stranded sequences. The result of this experiment is shown in figure 10 lane 3 (no gel denaturation) and demonstrates the presence of material having the same mobility as monomeric and dimeric molecules; these molecules might, thus, be partially single stranded. After denaturation of the gel, (lane 2)

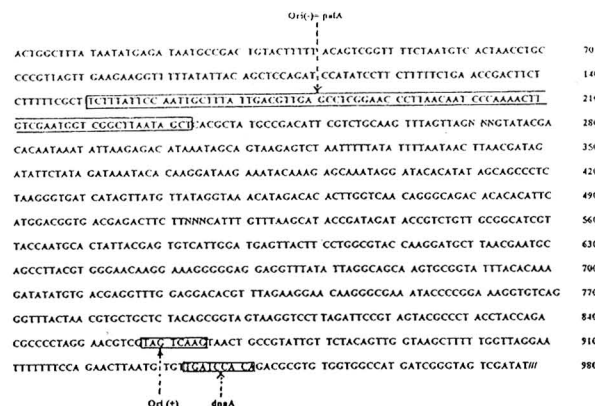


Fig. 9. DNA sequences of pCMA/HaeIII restriction fragment B (about 1000 bp) and homologies found (in boxes).



Fig. 10. Evidence for the presence of single-stranded material in pCMA. Lane 1: Agarose gel electrophoresis of total lysate of *B. subtilis* carrying pCMA, seen under UV light. Lane 2: ECL revelation of hybridization with a pCMA probe, after Southern-blot of the gel prior denaturation. Lane 3: The same as in lane 2 but without denaturation.

double stranded monomeric and dimeric molecules were revealed, as well as multimeric forms which are faintly visible under UV light (line 1). In the three lines, a small quantity of fast-moving material is visible which disappeared after S1 nuclease treatment (result not shown). This could be single stranded monomeric plasmid molecules.

CONCLUSION

We can conclude that pCMA replicates in *B. subtilis* as a result of the insertion of phage sequences in a pUC19 derived replicon-probe plasmid, as was shown earlier with other replicon-probes plasmids (3). The analysis of the replicative functions of the recombinant plasmid is complicated by the rearrangements suffered by the hybrid molecules upon transformation of *B. subtilis*. We found that this recombinant plasmid has lost all of the pUC19 sequences, as well as its ability to replicate in *E. coli*. A deletion derivative pCMB was obtained still able to replicate in *B. subtilis*, yet with a reduced segregational stability. The deletion in pCMB concerned essentially phage sequences, which thus might contain stability functions. We tried to prove the presence of single-stranded DNA intermediates in the plasmid replication; this was displayed on a Southern blot of the gel not having been treated with alkali. The pCMA probe revealed molecules having

the same mobility as double-stranded monomers and dimers, suggesting that these molecules contain sufficient single-stranded regions to be retained on the nylon membrane, without gel denaturation. Plasmid pCMA might thus replicate via a rolling circle mechanism, but with a very efficient system for complementary strand synthesis. On the other hand, physical mapping and hybridization with 2C DNA allowed the localization of the plasmid sequences of phage origin and the sequencing revealed the presence of 350 bp, identical to the N-terminal part of the *tet* gene of pBR322. In this plasmid, the corresponding 98 amino acids are responsible for membrane binding of the plasmid, providing supercoiling and perhaps stability. We found also in this fragment a *dnaA* box. Other interesting results found in the analysis of the sequences were the sequence containing the (-) origin of pC194 provided with the fragment carrying the *Cm*-resistance gene as well as the high homology found with the (+) origin of phage ϕ X174 and plasmids pUB110, pBAA1 and pC194. We can infer, according to the results described above, that we could be in the presence of a new member of Gram-positive plasmids that replicate via single-stranded DNA, but the replication and stability functions of this plasmid must be deeply studied in order to establish by subcloning specific parts in a replicon probe plasmid, which are the minimal regions responsible for driving both replication and stability functions. Experiments are under way to analyze these topics.

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