Structural organization of the *Corynebacterium glutamicum* plasmid pCG100

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(Received 18 January 1991; revised 30 May 1991; accepted 6 June 1991)

pCG100, a 3 kb cryptic plasmid of *Corynebacterium glutamicum* ATCC 13058, probably identical with pSR1 from *C. glutamicum* ATCC 19223, was characterized. The minimum region for autonomous replication was shown to be contained on a 1.9 kb *Bgl*II–*Nco*I fragment; a 380 bp *Hind*III–*Sph*I fragment can replicate in the presence of the parental plasmid, which presumably provides a *trans*-acting replication factor. Derivatives of pCG100 are able to replicate in several *Corynebacterium*, *Brevibacterium* and *Arthrobacter* strains. pCG100 is compatible with pBL1, a cryptic plasmid of *Brevibacterium lactofermentum*. Shuttle plasmid vectors, containing the kanamycin-resistance gene from Tn903 or from *Streptococcus faecalis* as selectable markers and the Amp^R, Tet^R or *lacZ* α genes for insertional inactivation, were constructed using the minimum replication fragment of pCG100.

Introduction

Corynebacteria are pleomorphic asporogenous Gram-positive bacteria widely distributed in nature. Non-pathogenic coryneforms are industrially used to produce amino acids and nucleotides (Bousfield & Calley, 1978).

Several cryptic plasmids have been found in corynebacteria (Martin, 1989). Two of them have been used to construct cloning vectors: pBL1, a 4.4 kb *Brevibacterium lactofermentum* plasmid (Miwa *et al.*, 1985; Santamaria *et al.*, 1985) and pSR1, a 3 kb plasmid isolated from *Corynebacterium glutamicum* (Yoshihama *et al.*, 1985). The nucleotide sequence of pBL1 has been published (Filpula *et al.*, 1986); that of pSR1 was determined and its open reading frames described (Archer *et al.*, 1989), but the nucleotide sequence remains to be published.

This report describes a 3 kb plasmid isolated from *C. glutamicum* ATCC 13058 which is very similar and possibly identical to pSR1 (Yoshihama *et al.*, 1985), pHM1519 (Miwa *et al.*, 1985) and pCG1 (Ozaki *et al.*, 1984), isolated from *C. glutamicum* ATCC 19223, 13058 and 31808, respectively.

Methods

Bacterial strains and plasmids. The coryneform strains screened for the presence of small plasmids were *C. glutamicum* ATCC 21650, ATCC 21488, ATCC 21649, ATCC 21652, ATCC 21573 and ATCC

13058. The latter was used as the source of the plasmid pCG100 studied in this work.

B. lactofermentum ATCC 21086, which carries pBL1 (Yeh *et al.*, 1986), was used for pCG100/pBL1 incompatibility studies. *Arthrobacter aureus*, *Arthrobacter simplex*, *Arthrobacter ureafaciens* (Professor A. M. Gounot, Université Claude Bernard, Villeurbanne, private collection) and *C. glutamicum* ATCC 13287 were used to test pCG100 replication.

Escherichia coli NM522 (*supE thi* $\Delta(lac-proAB)$ $\Delta(hsdMS-mcrB)$ 5 F⁺[*proAB*, *lacI*^Q, $\Delta lacZM15$]) (Mead *et al.*, 1985) was used as the recipient for transformation experiments and *E. coli* K38 (Russel & Model, 1984), harbouring pGP1-2, for gene expression studies (Tabor & Richardson, 1985).

E. coli plasmids used for cloning and vector construction were as follows: pBS8 (Spratt *et al.*, 1986), pJRD16905 (this work), pSU19 and pSU20 (chloramphenicol resistance vectors derived from pSU2719 and obtained from F. de la Cruz: Martinez *et al.*, 1988); pT7-5 and pT7-6 were used to identify pCG100-encoded polypeptides in *E. coli* (Tabor & Richardson, 1985). pHP45 Ω Cm (Fellay *et al.*, 1987) was used as source of the Ω cartridge containing the chloramphenicol (Cm) resistance gene of pKT210. pJRD16905 and p13313 (Fig. 1) were specifically constructed for this work. These plasmids and pBS8 (see above) contain kanamycin-resistance determinants which can be selected for in *E. coli* and in corynebacteria.

Growth conditions. All strains were grown on L-broth (g l⁻¹: Bactotryptone, 10; yeast extract, 5; NaCl, 5; pH 7.3) at 34 °C for coryneform strains and 37 °C for *E. coli* strains unless stated otherwise. For cloning experiments using pBS8, *E. coli* NM522 transformants were selected on MacConkey lactose plates (Miller, 1972). Kanamycin, ampicillin and chloramphenicol were used at 50 μ g ml⁻¹ and tetracycline at 10 μ g ml⁻¹. Only kanamycin (at 50 μ g ml⁻¹) was used for the selection of coryneform strains.

Manipulation of plasmid DNA. *E. coli* plasmid extraction was achieved by the alkaline extraction procedure described by Birnboim & Doly (1979). Plasmids from coryneform bacteria were extracted by a cleared-lysate technique. Cultures grown in 400 ml L-broth to an

Abbreviation: MCS, multiple cloning site.

Fig. 1. Construction of the replication-probe vectors pJRD16905 and p13313. We constructed kanamycin replication-probe vectors based on the *E. coli* Amp^r Tet^r plasmid pJRD184 (Heusterspreute *et al.*, 1985). The kanamycin-resistance gene (*aphIII*) of *Streptococcus faecalis* was isolated on a BssHII-RsaI fragment from pAT21-1 (Trieu-Cuot & Courvalin, 1983) and inserted between the MluI and PvuII sites of pJRD184, yielding pJRD16905. p13313 was constructed by inserting the MluI fragment of pBS8 containing the Tn903 neomycin-resistance gene (*neo*) at the MluI site of pJRD184. These plasmids enable kanamycin selection in corynebacteria and contain numerous cloning sites, most of which are located in the ampicillin- (*bla*) or tetracycline- (Tet^r) resistance genes, therefore permitting easy recombinant plasmid selection and screening in *E. coli*. With the exception of EcoRV for pJRD16905 and XhoI, MluI and ClaI for p13313, all the sites represented are unique and could be used for cloning.

OD₅₇₀ of 0.6 were treated with 0.25 µg penicillin (Serva) ml⁻¹. When the OD₅₇₀ reached 1.2, cells were harvested, washed once with 10 ml 10 mM-Tris/HCl buffer pH 8 and frozen at -80 °C for at least 1 h. Thawed cells were then resuspended in 4 ml 10 mM-Tris/HCl, 10 mM-EDTA, 10.3% (w/v) glucose, pH 8, containing 10 mg lysozyme ml⁻¹ and incubated at 37 °C for 60–120 min with shaking. After addition of 0.4 ml 10% (w/v) SDS, the suspension was thoroughly mixed, incubated for 20 min at 60 °C and cooled on ice for 5 min. NaCl was added to 0.5 M final concentration and incubation on ice was continued for at least 4 h. The plasmid-containing supernatant was collected by centrifugation at 30000 g for 20 min. All plasmids were further purified by dye-buoyant centrifugation in CsCl gradients containing ethidium bromide (Maniatis *et al.*, 1982).

Restriction endonuclease digestions and ligations using T4 DNA ligase were performed as recommended by the manufacturer. DNA analysis was carried out on agarose gels.

Transformation. *E. coli* cells were prepared for transformation with plasmid DNA by the method of Mandel & Higa (1970).

All coryneform strains were electrotransformed by using a Gene Pulser equipped with the Pulse Controller attachment (Bio-Rad). The method used was that described by Bonnassie *et al.* (1990), slightly modified. Mid-exponential-phase cultures (OD₅₇₀ 0.5) were treated with 0.25 µg penicillin ml⁻¹. When an OD₅₇₀ of 1 was reached, cells

were harvested, washed twice with 0.1 culture volume of 0.5 M-sucrose, resuspended in 0.01 culture volume of 0.5 M-sucrose and kept on ice until electroporation was done. For electroporation, 0.2 ml of concentrated cells was mixed with DNA and immediately introduced into a pre-cooled 2 mm cuvette and pulsed with instrument settings of 25 µF, 2.5 kV and 400 Ω. The time constant was between 5 and 7 ms. The cell suspension was immediately diluted with 1 ml L-broth and incubated at 34 °C for 90 min before selection of transformants on kanamycin-containing L-agar plates.

Gene expression in the bacteriophage T7 promoter-RNA polymerase system. A two-plasmid system based on the properties of the T7 RNA polymerase was chosen to study pCG100-encoded polypeptides. A number of overlapping pCG100 fragments were cloned downstream of the strong T7 Φ10 promoter, in the two possible orientations with respect to Φ10, depending on the use of pT7-5 or pT7-6 (Tabor & Richardson, 1985). The hybrid plasmids were introduced into *E. coli* K38, which contained the compatible plasmid pGP1-2, encoding T7 RNA polymerase under the control of the inducible λP_L promoter and the gene encoding the heat sensitive λ repressor cI857.

To preferentially label the products of the genes cloned downstream of Φ10, a slight modification of the protocol of Tabor & Richardson (1985) was used. Thermal induction was performed for 20 min at 43.5 °C, rifampicin was then added to 600 µg ml⁻¹ and cells were

incubated for an additional 20 min at the same temperature followed by 30 min at 30 °C. Then 10 µCi (370 kBq) L-[³⁵S]methionine (specific activity > 1000 Ci mmol⁻¹) was added and incubation was continued for 7 min. Samples were submitted to SDS-PAGE 12.5% (w/v) acrylamide with the discontinuous buffer system of Laemmli (1970). Size standards were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, α-lactalbumin, whose molecular sizes are 94, 67, 43, 30, 20.1 and 14.4 kDa respectively (Pharmacia). The gel was stained with Coomassie blue, dried and autoradiographed.

Results

Isolation of pCG100

In order to obtain a plasmid able to replicate in *C. glutamicum* and *B. lactofermentum*, and also compatible with pBL1-derived vectors, we searched for plasmids in our *C. glutamicum* strain collection. We found 3 kb plasmids in strains ATCC 13058, ATCC 21560, ATCC 21488 and ATCC 21649. The 3 kb plasmids from all four strains gave the same DNA fragments when analysed by restriction enzyme cleavage, and the restriction map of pCG100 from strain ATCC 13058 (Fig. 2) was similar to that previously described for pSR1 (Yoshihama *et al.*, 1985). pCG100 and pSR1 may be identical because they were both isolated from descendants of *C. glutamicum* ATCC 13032.

Identification of the minimal replication region of pCG100

Since kanamycin resistance generally constitutes a good selection marker for coryneform bacteria, we used the *E. coli* plasmids pBS8 (Spratt *et al.*, 1986) and pJRD16905 (Fig. 1), containing the Tn903 neomycin-resistance gene (*neo*) and a kanamycin-resistance gene (*aphIII*) of *Streptococcus faecalis*, respectively, to test the ability of cloned pCG100 fragments to promote replication in corynebacteria.

pBS8 and pJRD16905 were used to clone the entire pCG100 sequence as shown in Fig. 2. pSRB1 and pSRB2 contain *Bcl*I-linearized pCG100 in the *Bam*HI site of pBS8 in opposite orientations. pSRF1 contains *Bgl*II-linearized pCG100 in the *Bam*HI site of pJRD16905.

pSRB1, pSRB2 and pSRF1 were able to replicate both in *E. coli* and in the coryneform strains, *C. glutamicum* ATCC13287, *B. lactofermentum* ATCC 21086, ATCC 6872, *A. ureafaciens*, *A. aureus* and *A. simplex*. Plasmids isolated from these kanamycin-resistant transformed strains were of the expected sizes; no alteration of plasmid structure was observed after restriction enzyme cleavage. This indicated that both the *Bgl*II and the *Bcl*I sites of pCG100 are in regions non-essential for plasmid replication.

pSRF 2, pSRF3 and pSRF4 were constructed by the insertion into pJRD16905 of the 2.5 kb *Bcl*II–*Sca*I, 2.2 kb *Bcl*I–*Nco*I and 2 kb *Bgl*II–*Nco*I fragments of pCG100 respectively (Fig. 2). These deletion derivatives of pCG100 were able to replicate autonomously in *B. lactofermentum* ATCC 21086 and *A. aureus* lacking pCG100. The 1.9 kb *Bgl*II–*Nco*I fragment found in pSRF4 is the smallest pCG100 fragment allowing autonomous replication.

All the above self-replicating plasmids, when introduced by transformation into *C. glutamicum* ATCC 13058, caused the loss of the resident pCG100, which lacks a resistance marker.

Localization of *oriV*

pSRB3 contains the entire pCG100 sequence interrupted at the *Eco*RI site; pSRB4, pSRB1ΔE and pSRB3ΔB/*Bgl*II contain the 2.75 kb *Hind*III, the 1.8 kb *Eco*RI–*Bcl*I and the 1.6 kb *Eco*RI–*Bgl*II fragments of pCG100, respectively. These four constructions and pSRB1/ΩCm could replicate only in the presence of pCG100. Cointegrate formation was not detected when the plasmids were extracted from *C. glutamicum* ATCC 13058 and probed with a DNA fragment of pCG100 absent in these derivatives or when the plasmids were analysed by their restriction pattern. In all cases we observed the structure of the native pCG100 derivative. These results indicated the existence of *trans*-acting pCG100 replication factors.

pSRF1ΔH, pSRF4ΔE and pSRF5 (Fig. 2) contain the 0.2 kb *Hind*III–*Bgl*II, the 0.35 kb *Eco*RI–*Nco*I and the 2.05 kb *Sph*I–*Eco*RI segments of pCG100 respectively. They were unable to replicate even when pCG100 was provided *in trans*. It therefore seems likely that *oriV* is located on a 0.38 kb fragment between *Hind*III_b and *Sph*I. It does not overlap these sites since plasmids containing pCG100 deleted of its small *Hind*III fragment (pSRB4, Fig. 2), or with an insertion in the *Sph*I site (pSRB1/ΩCm, Fig. 2) could still replicate with the help of pCG100.

Compatibility with pBL1

The six self-replicating kanamycin-resistant pCG100 derivatives shown in Fig. 2 were maintained in *B. lactofermentum* ATCC 21086 together with pBL1, at about equal copy number. This suggests that pCG100 and pBL1 are compatible replicons.

Copy number

The copy number of pCG100 was estimated by comparing the amount of plasmid DNA in the covalently closed circular form with the amount of chromosomal DNA in

Fig. 2. Derivatives of pCG100 and their properties. pCG100 (top of Figure) was linearized at the *Bcl*I site and inserted in both orientations into the *Bam*HI site of pBS8 to give pSRB1 and pSRB2 respectively. pSRB3 and pSRB4 were obtained after introduction of *Eco*RI- or *Hind*III-linearized pCG100, respectively, into the corresponding sites of pBS8. Insertion of *Bgl*II-cleaved pCG100 into the *Bam*HI site of pJRD16905 gave pSRF1. pSRF2, pSRF3 and pSRF4 were obtained by the introduction of the *Bcl*I-*Sca*I (2.6 kb), *Bcl*I-*Nco*I (2.1 kb) or *Bgl*II-*Nco*I (1.9 kb) fragment, respectively, between *Bam*HI and the appropriate second sites of pJRD16905. pSRF5 was obtained by inserting the *Eco*RI-*Sph*I fragment (0.95 kb) between the corresponding sites of pJRD16905. pSRB1/ΩCm resulted from the insertion of the Ω cartridge, containing a chloramphenicol-resistance gene, into the *Sph*I site of pSRB1. pSRB1ΔE, pSRB2ΔE, pSRB3ΔB/Bg, pSRF1ΔH and pSRF4ΔE were derived by deletion of the indicated fragments from the named plasmids. All plasmids were first isolated from *E. coli* NM522 and then transformed by electroporation into corynebacteria. Only the cloned pCG100 fragments are shown. The position of relevant vector sites is indicated without scale considerations. The positions of the minimal replicon and replication origin (oriV) are shown on the pCG100 map at the top of the Figure. pSRB3 is represented linearized at its *Bcl*I site; the position and orientation of pBS8 is shown above the pSRB3 line. *E. coli* cloning vectors are specified in the first column to the right. The column 'Aut. rep.' indicates whether the plasmids can replicate autonomously in *B. lactofermentum* and *A. aureus*. The rightmost column indicates the ability of fragments to sustain replication in *C. glutamicum* ATCC 13058 containing pCG100: + indicates that both replicons coexist; INC indicates incompatibility, i.e. resident pCG100 is lost. B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III (*H_a* and *H_b* denote the two *Hind*III sites of pCG100); N, *Nco*I; P, *Pst*I; S, *Sal*I; Sc, *Sca*I; Sm, *Sma*I; Sp, *Sph*I.

total DNA preparations obtained according to Chater *et al.* (1982). Assuming that the size of chromosomal DNA of corynebacteria is around 3000 kb (Crombach, 1978), the copy number of pCG100 is about 30 in *C. glutamicum*

ATCC 13058 and in *B. lactofermentum* ATCC 21086. In the latter strain we found that pCG100 derivatives had the same copy number as pBL1. This result agrees with the estimation of Santamaria *et al.* (1984).

Fig. 3. Synthesis of pCG100 polypeptides in the T7 expression system of Tabor & Richardson (1985). (a) Plasmid constructs. pCG100 was linearized with *Bcl*I and ligated to *Bam*HI-linearized pT7-5 to give p21917 and p21958, which differ in the orientation of pCG100 with respect to the Φ 10 promoter; p21912 and p21964 were constructed with *Bam*HI-linearized pT7-6. All plasmids were introduced by transformation into *E. coli* K38 for analysis as described in Methods. The sizes of the polypeptides expressed from each plasmid are indicated on the right (see also b). The figure shows only the pCG100 fragments cloned into the pT7 plasmids. The arrow indicates the direction of transcription from the Φ 10 promoter. CS1 and CS2 cloning sites are detailed on the left and on the right of p21917 respectively. Sa, *Sac*I; X, *Xba*I; other restriction endonucleases as in Fig. 2. (b) Autoradiograph of SDS-PAGE gels showing [^{35} S]methionine-labelled polypeptides encoded by the cloned pCG100 sequences. Lanes: A, p21917; B, p21917ΔE; C, p21917ΔH; D, p21964ΔE; E, p21912; F, p21912ΔE; G, p21912ΔH; H, p21958ΔE; I, p21958ΔH. The positions of molecular mass markers are shown in the centre. On the left and right sides are indicated the molecular masses of the pCG100-encoded polypeptides which depend on the direction of transcription analysed. Lanes A–D and E–I were exposed for 16 h and 2 d respectively. The locations of the pCG100 sequences encoding the polypeptides, and the minimal replicon are shown reported at the top of (a).

Fig. 4. Construction of *E. coli*/*Corynebacterium* shuttle vectors based on the pCG100 minimal replication region. (a) pCG100 digested with *NcoI* and *BclI* was ligated to pJRD16905 cleaved with *BamHI* and *NcoI*. The ligation mixture was used to transform *A. aureus*, a strain devoid of resident pCG100 and suitable for pCG100 replication. The resulting plasmid, p25400, was digested with *BglII* and *XhoI* and the DNA fragment containing both the kanamycin-resistance gene (*aphIII*) and the pCG100 replication region was ligated to pJRD184 cleaved with the same enzymes. The resulting plasmid, p25427, conferred an Amp^rTet^rKan^r phenotype upon *E. coli* NM522. (b) *BglII*-cleaved pCG100 was introduced into the unique *BglII* site of p13313, to give p25401, which was selected in *A. aureus*. (The other orientation of pCG100 in p13313 was also obtained.) The *NcoI* fragment bearing the neomycin resistance gene (*neo*) and the pCG100 replication functions was ligated to pSU19 or pSU20 linearized at their unique *NcoI* sites to give p25435 and p25450, respectively. The orientations of the MCS are indicated by an arrow on the plasmid maps and on the polylinkers.

Identification of pCG100-encoded polypeptides

The entire pCG100 sequence linearized with *Bcl*I was introduced into pT7-5 and pT7-6 in the same orientation with respect to Φ 10 promoter activity, yielding p21917 and p21964, respectively (Fig. 3a). These two plasmids expressed three polypeptides, of 12.4, 18 and 32 kDa, the latter being the best labelled (Fig. 3b, data not shown for p21964). These polypeptides were encoded by genes transcribed from *Eco*RI towards *Hind*III. The other orientation of the pCG100 sequence with respect to the Φ 10 promoter was found in p21912 and p21958, in which *Bcl*I-linearised pCG100 was cloned in pT7-6 and pT7-5 respectively. These two plasmids gave rise to the synthesis of two polypeptides of 14.2 and 16 kDa (Fig. 3b, data not shown for p21958).

To localize more precisely the sequences necessary for the synthesis of these polypeptides, deletion derivatives of the above four plasmids were analysed (Fig. 3). p21917 Δ H still expressed the three polypeptides, whereas p21917 Δ E expressed only the 32 kDa polypeptide and p21964 Δ E expressed those of 12.4 and 18 kDa. Hence, we conclude that the major polypeptide of 32 kDa is expressed from the sequence between the *Eco*RI and *Hind*III_b sites of pCG100 and that the other two polypeptides are transcribed on the same DNA strand from a sequence located between *Bcl*I and *Eco*RI.

Considering the other direction of transcription, p21912 Δ E expressed only the 14.2 kDa polypeptide, whereas p21912 Δ H expressed only the 16 kDa polypeptide. Since these two p21912 derivatives overlap on the *Eco*RI–*Hind*III_b sequence, it was obvious that the 14.2 kDa polypeptide was encoded between the *Bcl*I and *Hind*III_b sites, whereas the 16 kDa polypeptide was encoded between *Eco*RI and *Bcl*I, as confirmed by p21958 Δ E analysis (Fig. 3).

The localization of the pCG100 sequences encoding the polypeptides is summarized at the top of Fig. 3.

Shuttle vectors derived from pCG100

Three shuttle vectors based on the *Bgl*II–*Nco*I fragment containing the minimum pCG100 replication region were constructed (Fig. 4). p25427 (6.7 kb) contains the entire Tet^R and Amp^R (*bla*) genes, the ColE1 origin of replication of pJRD184 and the *aph*III gene of *Streptococcus faecalis*. p25435 and p25450 (each 5.9 kb), derived from pSU19 and pSU20, respectively, contain the P15A replication origin, the Tn903 neomycin resistance gene and the *lacZ* α reporter gene of pSU19 or pSU20 respectively. These three vectors contain numerous unique cloning sites, most of them enabling resistance gene inactivation for recombinant plasmid selection in p25427 (*Nru*I, *Sal*I, *Bam*HI, *Cla*I, *Xmn*I, *Sca*I and *Pvu*I in

the Amp^R or Tet^R genes and unique *Bgl*II, *Bcl*I, *Nde*I, *Nsi*I, *Apa*I, *Sma*I, *Hpa*I, *Mlu*I and *Sac*II sites in the MCS), or direct selection of insert-containing plasmids on MacConkey lactose kanamycin plates for p25435 and p25450 (both contain unique *Sal*I, *Xba*I, *Bam*HI, *Kpn*I and *Sac*I sites in their MCS, and p25435 additionally contains *Pst*I; *Nde*I, *Nsi*I and *Bgl*II are unique sites that could also be used for cloning).

Discussion

The cryptic 3 kb multicopy plasmid pCG100 found in *C. glutamicum* ATCC 13058 has a restriction map similar to that described for pSR1 (Archer *et al.*, 1989), but the location on the pCG100 map of the region necessary for autonomous replication and of the sequences encoding polypeptides (see below) seem different from pSR1 described by Archer *et al.* (1989).

To identify pCG100-encoded proteins we used the *E. coli* expression system described by Tabor & Richardson (1985). In this system, cloned genes are expressed from the Φ 10 promoter while transcription from other promoters is inhibited by rifampicin. This method overcomes problems of heterologous promoter expression in *E. coli*. Various *Corynebacterium* genes have been cloned and expressed in *E. coli* (Martin, 1989); in some cases the original promoter was not effective in *E. coli*. The translation signals of known *Corynebacterium* genes are similar to those of *E. coli*, whereas their codon usage is slightly different (Martin, 1989). These differences do not seem to be very important since most of the amino-acid-producing genes cloned from corynebacteria were identified by complementation of *E. coli* mutants (Martin, 1989).

We have identified five polypeptides encoded by pCG100. Their molecular sizes are 12.4, 18, 32, 14.2 and 16 kDa; the first three were expressed on one of the DNA strands, the latter two on the other strand. The 32 kDa polypeptide gave rise to the strongest bands in the T7 expression system.

Deletion experiments enabled us to localize the 32 kDa polypeptide-coding sequence between the *Eco*RI and *Hind*III_b sites (Fig. 3). The polypeptides of 12.4 and 18 kDa, which are dispensable for pCG100 replication and expressed on the same DNA strand, are located between the *Bcl*I and *Eco*RI sites. Considering the other direction of transcription, only two very weakly labelled polypeptides of 14.2 and 16 kDa were expressed. The 16 kDa protein originates from between the *Eco*RI and *Bcl*I sites and the 14.2 kDa protein from between *Bcl*I and *Hind*III_b (Fig. 3).

The role of these polypeptides is not known. However, since the 14.2 kDa protein is absent and the 18, 16 and

12.4 kDa ones are truncated in the self-replicating plasmid pSRF4, these polypeptides are dispensable for autonomous replication of pCG100. The 32 kDa protein, in contrast, is absolutely required for self-replication since insertion at the *Sph*I site abolished autonomous replication. It is tempting to assume that the 32 kDa protein is the replication protein. Its molecular mass is in agreement with that of other Gram-positive replication proteins, which vary from 25 to 40 kDa (del Solar *et al.*, 1987; Khan *et al.*, 1988; Byeon & Weisblum, 1990).

Deletions starting at *Eco*RI and extending to the *Nco*I and *Bcl*I sites (pSRB1ΔE, pSRB3ΔB/Bg) affected self-replication, but not the production of the 32 kDa protein in the pT7 system. pSRF4, containing the 1.9 kb *Bgl*II–*Nco*I fragment, is able to replicate autonomously. Since no origin of replication was detected in this region, it is likely that the *Eco*RI site is located within the promoter region or between the promoter and the coding sequence for the presumptive replication protein.

Some derivatives of pCG100 could replicate only when *trans*-complemented by pCG100. They all contained the 0.4 kb *Sph*I–*Hind*III_b fragment which should contain the replication origin of pCG100. It is located within or close to the 3' end of the 32 kDa presumptive replication protein gene.

The *Hind*III_b–*Bgl*II segment downstream of the sequence coding for the 32 kDa protein and the *cis*-acting replication origin sequence is required for autonomous replication. This segment, of less than 200 bp, is too short to encode the 14.2 kDa polypeptide located in this region, making it unlikely that this polypeptide is involved in pCG100 replication. No other polypeptide was detected in this region, so it is tempting to speculate that an RNA encoded in this region may be necessary for pCG100 replication.

Derivatives of pCG100 containing the minimal autonomous replication region are incompatible with pCG100. This incompatibility results either from a control of plasmid copy number, as observed for many replicons (Scott, 1984), or from competition for the replication protein (Novick, 1989). Since pCG100 is compatible with pBL1, the replication mechanisms of these two plasmids do not interact, proving that they are two distinct replicons.

The data presented in this report locate the replication origin of pCG100 and identify the sequence for a 32 kDa putative replication protein. The characterization of the minimal replication region allowed the construction of new shuttle vectors for cloning in most corynebacteria since pCG100 seems to have a wide host spectrum. The precise identification of the replication mechanisms will require the purification of the various components, including the replication protein, in order to develop an *in vitro* assay as described for pT181 (Gennaro *et al.*,

1989) and pMU158 (del Solar *et al.*, 1987). This should be possible, since the presumptive replication protein is highly expressed in the pT7 expression system.

Three new *E. coli*–*Corynebacterium* shuttle vectors were constructed. They contain either the P15A or the ColE1 replication origin for *E. coli* and the 1.9 kb minimal region necessary for autonomous replication in corynebacteria. They present original features in comparison with those already described (Martin, 1989), namely numerous cloning sites enabling resistance gene or *lacZ*α inactivation detectable in *E. coli*. In corynebacteria, the *E. coli lac* genes are expressed from their own promoter, this expression being controlled by the *lacI* gene product (Tsuchiya & Morinaga, 1988). Thus a β-galactosidase α complementation system for insert detection in corynebacteria may be feasible after introduction of *lacZ*β on a pBL1 derivative which is compatible with pCG100. Such a strategy was successfully used in *Bacillus subtilis* (Haima *et al.*, 1990).

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