LOCALIZATION OF THE STRUCTURAL GENE OF COLICIN A ON THE RESTRICTION MAP OF THE PLASMID pCol A–CA 31 THROUGH HYBRIDIZATION WITH ITS MESSENGER RNA

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1. Introduction

Colicinogenic plasmids encode various proteins amongst which colicins and immunity proteins are specific to the plasmid. Colicins are toxins for related species of *Escherichia coli* and immunity proteins confer protection against these toxins to the plasmid host cell. Like the colicinogenic plasmid Col E1 (pCol E1), the colicinogenic plasmid Col A (pCol A) is amplifiable in the presence of chloramphenicol [1]. It thus could be used as a vector allowing production of bank of genes for various organisms [2]. The restriction map of pCol A has been made [1]. Localization of the various genes of this plasmid and in particular that of the structural gene for colicin A (*colicin A activity = caa*) has been undertaken.

Here, we describe the localization of *caa* gene through hybridization with the colicin A-specific mRNA. The isolation of this mRNA is based upon the fact that ~40 min after induction with mitomycin C, colicin A accounts for nearly 90% of total proteins produced in the strain *Citrobacter freundii* CA 31 [3].

2. Materials and methods

2.1. *Bacterial strain and plasmid*

The Col A plasmid was isolated from a strain constructed by A. Pugsley from a *nalA* derivative of *Escherichia coli* W3110 and the Col A plasmid originating from *Citrobacter freundii* CA 31. The Col A-mRNA was purified from *Citrobacter freundii* CA 31.

2.2. *Preparation of plasmid DNA*

The technique described in [4] was used. Plasmid was purified after chloramphenicol amplification from the strain W3110 Col A.

2.3. *Chemicals*

The commercial source of the following restriction endonucleases was Bethesda Research Labs. (MD): *Hin*dIII, *Sma*I, *Ava*I, *Pst*I, *Hin*cII. *Eco*RI λ phage DNA, used as calibration mixture, was purchased from Boehringer (Mannheim).

2.4. *Digestion of DNA with restriction endonucleases*

Buffers required for optimal activity of enzymes were described in the BRL catalogue. Plasmid DNA was digested for 4–8 h at 37°C.

Reactions were terminated by the addition of a solution containing 7 M urea, sucrose 50% (w/v), EDTA (0.1 mM) (pH 7.0) and bromophenol blue as tracking dye. When restriction fragments had to be used for a second digestion, the first incubation solution was deproteinized with chloroform and isoamylalcohol. Then the DNA restriction fragments were precipitated in Na-acetate 0.3 M and 2.5 vol. ethanol and kept for 15 min at −70°C. After centrifugation, the pellet was resuspended in a second digestion buffer and submitted to digestion.

2.5. *Agarose gel electrophoresis*

Plasmid DNA and plasmid fragments were resolved by electrophoresis on 0.8–1.5% agarose horizontal slab gels (20 × 20 × 0.4 cm) which were run for 3–4 h at 50 mA, using 90 mM Tris–HCl (pH 8.3); 32 mM EDTA; 90 mM borate as running buffer. Gels were stained for 30 min in aqueous ethidium bromide solution (5 μg/ml), destained for 15 min in water and photographed under short-wave ultraviolet light using

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a polaroid MP3 Land camera with red filter and Polaroid type 665 Land film. Size estimates of the plasmid fragments were based on the migration rate relative to endonuclease-generated fragments of λ/*Hin*dIII–*Eco*RI [5] assuming a logarithmic relationship between size and electrophoretic mobility and an av. M_r of 650 for a nucleotide pair.

2.6. *Localization of the Col A-structural gene*

Col A restriction fragments were immobilized on nitrocellulose filters and used for hybridization with ^{32}P-labelled Col A-mRNA as described.

3. Results and discussion

3.1. *Preparation of ^{32}P-labelled Col A mRNA from free polysomes*

The technique in [3] to prepare free polysomes that can be used to program cell-free colicin A synthesis, was used. This technique was slightly modified to allow ^{32}P incorporation in the Col A-mRNA. Briefly, free polysomes were extracted from the strain CA 31. Cells were grown at 37°C in 1 litre of minimal medium, supplemented with the appropriate nutritional requirements. When the absorbance at 600 nm reached 0.45, the culture was induced by addition of mitomycin C (0.1 μg/ml) for 40 min. The culture was centrifuged 5 min at 8000 × *g* and the pellet was suspended in 100 ml medium supplemented with the appropriate nutritional requirements but devoid of phosphate. $^{32}P_i$ (6 mCi) was added to the mixture before incubation at 37°C for 30 min. Cells were then harvested; from this point, the procedure was as in [3]. Free polysomes were suspended in 10 mM Tris–HCl (pH 7.6), 3 mM Mg-acetate, 60 mM NH_4Cl and kept at −70°C. ^{32}P-Labelled Col A-mRNA release was carried out by treatment of the free polysomes with 20 mM EDTA and 0.5% SDS. Deproteinization was achieved by addition of 2 vol. phenol. After gentle shaking, the aqueous phase containing ^{32}P-labelled Col A-mRNA was recovered, precipitated with 0.3 M Na-acetate and 2.5 vol. ethanol and kept at −20°C. In table 1, amounts of ^{32}P-incorporated that were recovered at each step are indicated. The restriction map of pCol A (M_r 4.7 × 10^6 or 7 kilobasepairs) recently determined [1] is presented in fig.1 in order to allow positioning of the *caa* gene.

Table 1

Steps	^{32}P Incorporated (× 10^6 cpm)
Cells	400
Free polysomes	10
mRNA	2.5
Hybridized mRNA	1.9

3.2. *Localization of the Col A structural gene*

To localize the structural gene of colicin A on the physical map, pCol A restriction fragments were hybridized with radiolabelled Col A-mRNA [6].

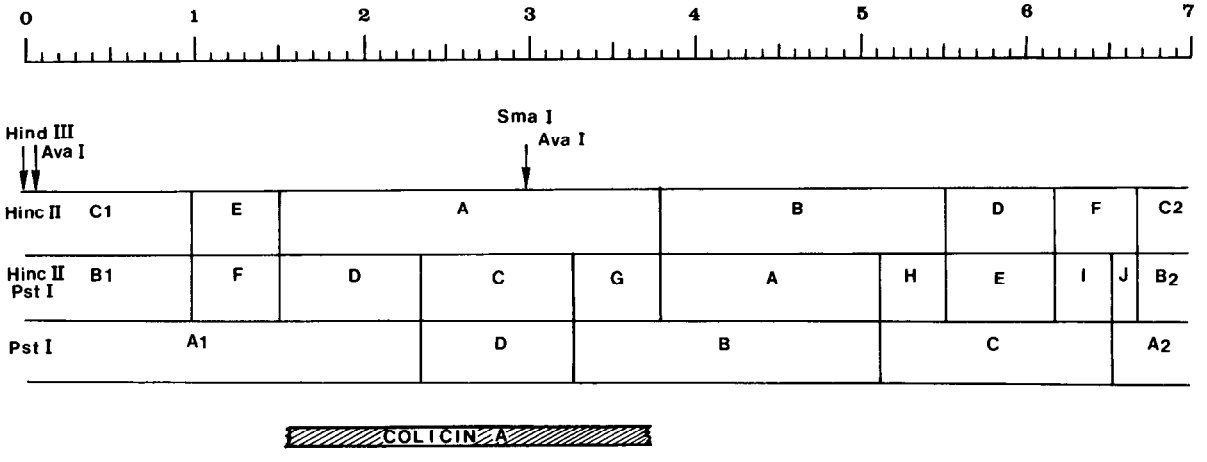

Fig.1. Restriction map of pCol A–CA 31. Physical coordinates are given in kilobasepair units (upper graduation). The arbitrary origin and terminus of the linear coordinate system is the single *Hin*dIII site. Sites for *Hin*cII, *Pst*I, *Ava*I and *Sma*I are shown in the boxes. The region of the map from 1.5–3.7 corresponds to the colicin A structural gene (shaded area).

As shown in fig.2 (lanes 6A and 6B), the maximum radioactivity was found in the 2.17 kilobasepair *Hinc*II fragment (A); the other *Hinc*II fragments were not labelled at all, indicating that all the colicin A structural gene was included in the 2.17 kilobasepair fragment. The fragments obtained with every other restriction endonuclease were labelled to various extents (fig.2) according to the part of Col A-structural gene they included. The proportion of labelling confirmed the physical map obtained in [1]. Among the *Pst*I fragments, the smallest (0.90 kilobasepair) (D) hybridized the most strongly but the 2.86 (A) and 1.80 (B) kilobasepair *Pst*I fragment also hybridized (fig.2, lanes 5A,5B); therefore these two fragments were contiguous to the smallest one (fig.1). Thus the *Pst*I fragments order, from position 6.50 on the map (fig.1) was: 2.86 (A), 0.90 (D), 1.80 (B), and 1.38 kilobasepairs (C).

All other hybridization results (fig.2) were consistent with the pCol A restriction map in [1]. The *caa*

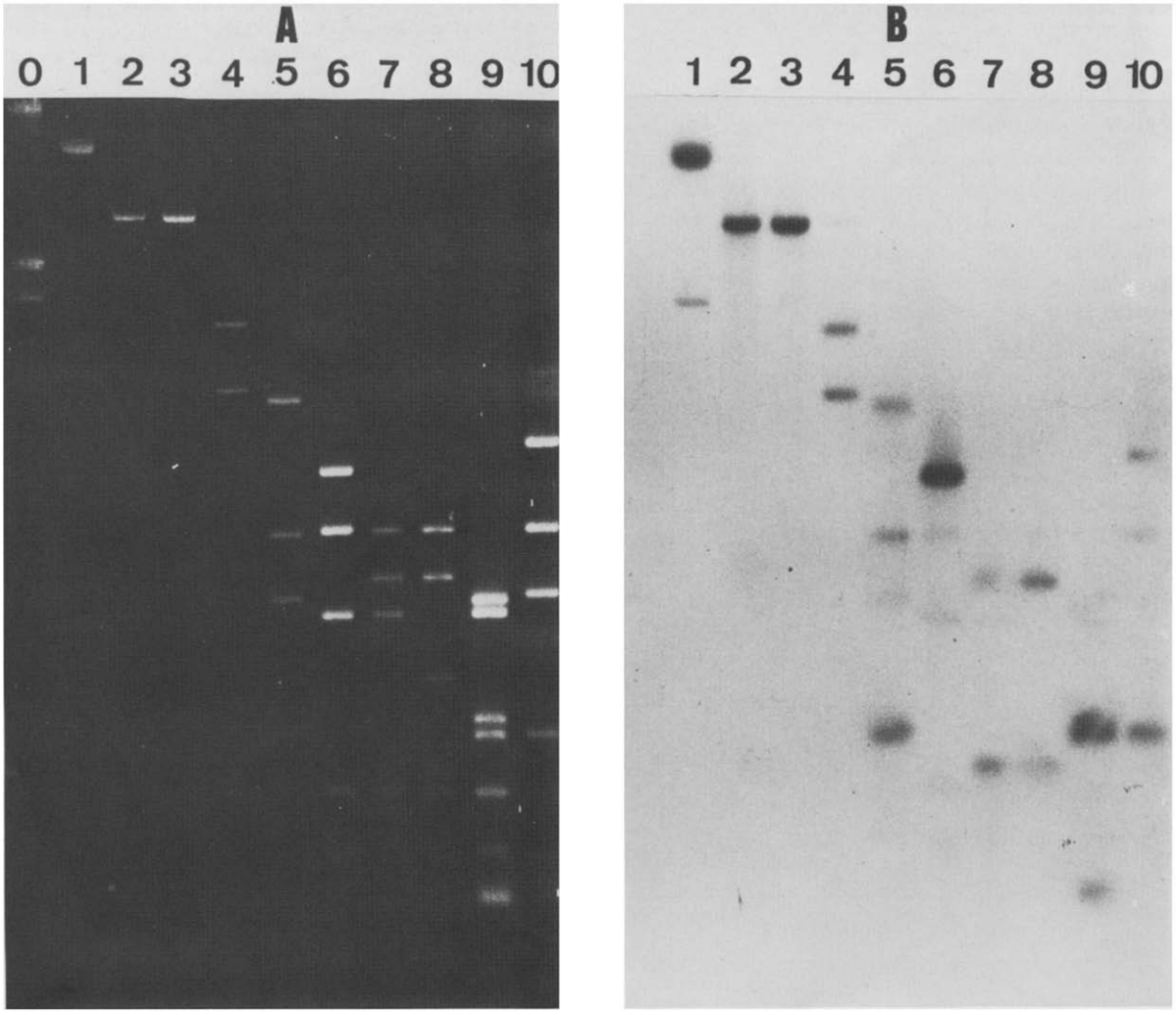

Fig.2. Hybridization between Col A-mRNA and pCol A digestion fragments. Plasmid DNA was digested with restriction endonucleases; resulting fragments were run on 0.8% agarose gel. After electrophoresis, fragments were stained and photographed. Digestion fragments were then transferred to nitro-cellulose paper and hybridized with radiolabelled Col A-mRNA: (A) stained pattern; (B) autoradiogram. Restriction endonucleases used for each digestion were: Lane (0) phage λ/*Hin*dIII + *Eco*RI (calibration mixture); (1) pCol A; (2) pCol A/*Hin*dIII; (3) pCol A/*Sma*I; (4) pCol A/*Ava*I; (5) pCol A/*Pst*I; (6) pCol A/*Hinc*II; (7) pCol A/*Hinc*II + *Sma*I; (8) pCol A/*Hinc*II + *Ava*I; (9) pCol A/*Hinc*II + *Pst*I; (10) pCol A/*Pst*I + *Hin*dIII.

gene accounts for 1.8–2.1 kilobasepairs and is entirely included in the 2.17 kilobasepair *Hin*cII pCol A fragment. It comprises 26–32% of the total pCol A. This percentage is almost the same as that occupied in the pCol E1 by the colicin E1 structural gene (*cea*), which represents 27% of the total pCol E1 [7]. Both colicins have a similar M_r, 56 000 for Col E1 [8] and 60 000 for Col A [3]. Therefore one could expect the size of the colicin A structural gene to be a little larger than that of colicin E1.

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