Isolation and Partial Characterization of Three Cryptic Plasmids from Strains of Clostridium butyricum

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Of seven strains of Clostridium butyricum examined, four contained covalently closed circular DNA molecules. The bacteriocinogenic strain C. butyricum NCIB 7423 carried two plasmids: pCB101 of molecular mass 3.9 megadaltons (5.6 x 10^3 bases) and pCB102 of molecular mass 5.2 megadaltons (7.4 x 10^3 bases). Plasmid pCB101 was not restricted by EcoRI which cleaved pCB102 at two sites; both plasmids were restricted by HindIII. The three other plasmid-bearing strains of C. butyricum (SA1, SA11 and NCTC 6084) each carried a single plasmid of molecular mass 4.3 megadaltons (6.2 x 10^3 bases) with a single cleavage site for EcoRI and four sites for HindIII, the fragments so produced from the three plasmids being indistinguishable on agarose gel electrophoretograms. It was concluded that the three strains harboured a common plasmid — pCB103.

INTRODUCTION

Comparatively little is known of the plasmids carried by species of Clostridium and of the possible range of plasmid-encoded functions in these obligately anaerobic Gram-positive bacteria. Both small [2 to 4 megadaltons (Md)] and large (18 to 25 Md) cryptic plasmids have been discovered in strains of C. botulinum (Scott & Duncan, 1978), but only in the case of several plasmids harbourd by strains of C. perfringens have functions been unequivocally assigned to these extrachromosomal elements.

A plasmid (pIP404) of 5.7 Md present in C. perfringens Type A, strain CPN50 was shown to specify production of, and immunity to, the perfringocin BC5 which is produced by this organism (Ionesco & Bouanchaud, 1973; Ionesco et al., 1976). Additionally, C. perfringens CPN50 harboured a large plasmid (pIP405) of 32.4 Md which was not associated with the production of the bacteriocin (Ionesco et al., 1976) though it might have played a role in the conjugative transfer of pIP404 to recipient strains (Brefort et al., 1977). Another bacteriocinogenic strain of C. perfringens, strain CW55, was similarly found to carry a small plasmid (pCW4) of 5.6 Md whose elimination coincided with loss of ability to produce the perfringocin and simultaneous loss of immunity to its bactericidal action (Mihelc et al., 1978).

Larger plasmids determining resistances to various antibiotics have been reported in strains of C. perfringens (Sebald & Brefort, 1975; Brefort et al., 1977, 1978), and Rood et al. (1978a) found that multiple antibiotic-resistant isolates of C. perfringens invariably contained one or more of such plasmids. Conjugal transfer of certain of these resistance plasmids to antibiotic-sensitive strains of C. perfringens has been reported (Sebald & Brefort, 1975; Rood et al., 1978b). The widespread occurrence of plasmids in toxigenic strains of C. perfringens has also been reported (Rokos et al., 1978; Duncan et al., 1978), together with evidence of the possible involvement of a large (75 Md) plasmid in the production of β toxin.

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cinogenic and non-bacteriocinogenic strains). No systematic study has, however, been undertaken of the distribution of plasmids in non-pathogenic species of butyric clostridia. It is the chief purpose of this communication to report the carriage of small plasmids by several strains of *C. butyricum* (both bacteriocinogenic and non-bacteriocinogenic strains).

**METHODS**

**Organisms.** *Clostridium butyricum* NCIB 7423, which produces the bacteriocin butyrin 7423 (Clarke et al., 1975), was obtained from the National Collection of Industrial Bacteria, Aberdeen. Non-bacteriocinogenic strains of *C. butyricum* were obtained as follows: strain NCTC 6084 from the National Collection of Type Cultures, London; strains SA1, SA11, CNRZ528 and CNRZ531 (Sarathchandra et al., 1974) from Mr J. W. Wolf, University of Leeds; strain 8558 from Dr T. A. Roberts, ARC Meat Research Institute, Langford, Bristol. Strains of *Escherichia coli* K12 which harboured known plasmids (indicated in parentheses) were obtained as follows: strains HB101 (pBR322) and AB2463 (pHV14) from Dr S. B. Primrose, University of Warwick; strain V517 (several pV517 plasmids; Macrina et al., 1978) from the Plasmid Reference Center, Stanford University, U.S.A.

All strains of *C. butyricum* were maintained at 4°C as sporulated cultures grown anaerobically on slopes of reinforced clostridial medium (RCM, Oxoid) solidified with 2% (w/v) agar. The plasmid-carrying reference strains of *E. coli* K12 were maintained on R broth medium (containing, per litre: tryptone, 10 g; yeast extract, 1 g; NaCl, 5 g; glucose, 1 g) solidified with 1-5% (w/v) agar.

**Growth of bacteria.** The strains of *C. butyricum* were grown anaerobically at 37°C in batch culture (150 ml to 5 l) in CYG medium (consisting of the glucose, ammonium, salts and vitamins minimal medium employed for *C. pasteurianum* (Robson et al., 1974) supplemented with, per litre: Casamino acids (Difco), 1 g; yeast extract (Oxoid), 3 g; L-cysteine. HCl, 0.5 g). Batch cultures (0.5 to 2 l) of the *E. coli* K12 strains were grown aerobically overnight at 37°C either in a commercial nutrient broth (CM1, Oxoid) or in R broth. In the case of the *E. coli* K12 strains carrying the ColEl plasmid derivatives pBR322 and pHV14, amplification of plasmid production was achieved by the addition of chloramphenicol (200 µg ml⁻¹) to cultures in the exponential phase of growth (absorbance at 650 nm of 0.7) followed by vigorous shaking at 37°C for 12 to 15 h.

**Plasmid screening.** Bacteria harvested from 150 ml of a culture of *C. butyricum* in CYG medium were washed in 20 ml TES buffer (50 mM-Tris/ HCl with 3 mM-EDTA plus 100 mM-NaCl, pH 8.0) and resuspended in 4 ml 50 mM-Tris/ HCl, pH 8.0 containing 25% (w/v) sucrose. The suspension was placed on ice for 10 to 15 min before 0.4 ml lysozyme (10 mg ml⁻¹, in 25 mM-Tris/ HCl, pH 8.0) was added. After vortex mixing, the suspension was held on ice for 10 to 15 min and 1.6 ml 250 mM-Na₂EDTA, pH 8.0 was then added. The suspension was gently mixed and held on ice for a further 10 to 15 min. Lysis was effected by adding 4 ml 2-5% (w/v) sodium dodecyl sulphate (SDS) in 50 mM-Tris/ HCl with 25 mM-Na₂ EDTA, pH 8.0. After gentle mixing the suspension was held on ice for 20 to 30 min and 2-5 ml 5 M-NaCl was then added. The lysate was stored overnight at 4°C, after which it was centrifuged at 17 000 g for 30 min at 4°C and the uppermost 80% of the resultant supernatant ('cleared lysate') was collected. Following treatment with ribonuclease I (20 µg ml⁻¹, final concentration) and extraction of protein, firstly with phenol and then with chloroform/3-methylbutan-1-ol (24:1, by vol.), the recombinant lysate (aqueous phase) was brought to 0.3 M-sodium acetate (final concentration) and 2 vol. chilled (−20°C) 95% (v/v) ethanol was then added to precipitate the DNA. The ethanol-precipitated DNA was dissolved in 0-2 to 0-4 ml TE buffer (10 mM-Tris/ HCl and 0.1 mM-Na₂EDTA, pH 7-0).

**Larger scale isolation of plasmid DNA.** Basically the same procedure was employed as above except that bacteria were harvested from 5 l of culture and Brij 58 was used together with SDS at the stage of cell lysis. Ethanol-precipitated DNA was further purified by caesium chloride buoyant density centrifugation as described by Colman et al. (1978).

**Agarose gel electrophoresis of plasmid DNA.** Vertical slab gels (0.6 x 9 x 14 cm) of 0.8 or 1.4% (w/v) agarose (Sigma) in 150 mM-Tris, 89 mM-boric acid, 2.5 mM-Na₂EDTA, pH 8.0 were employed. The plasmid DNA preparation was mixed with one-third of its volume of 0.1 M-Na₂EDTA containing 7% (w/v) SDS and 15 µg bromophenol blue ml⁻¹, and a sample of suitable size (<200 µl) was applied to the gel. Electrophoresis was at room temperature for 3.5 h at 100 V (64 mA). Gels were then soaked for 20 min in a fresh solution of ethidium bromide (0.5 µg ml⁻¹, in water) and viewed under ultraviolet light at 254 nm using a Chromato-Vue Transilluminator (model C-61, U.V. Products, San Gabriel, Calif., U.S.A.). DNA bands were photographed on Ilford FP4 film using a Hoya orange filter plus a Wratten 2B ultraviolet filter. Molecular masses of the clostridial plasmids were estimated from the relative mobilities of their open circular (oc DNA) forms alongside standard plasmid oc DNA bands, on electrophoresis in 0.8% agarose gels. For this purpose, the oc DNA bands yielded by five pV517 plasmids (molecular masses of 1-4, 1-8, 3-4, 3-7 and 4-8 Md) together with the monomer oc DNA...
bands of plasmid pBR322 (2.8 Md) and plasmid pHV14 (4.6 Md) were identified and employed to generate a linear regression plot of log relative mobility versus log molecular mass with a correlation coefficient of 0.999. The DNA contents of individual DNA bands were recovered by the agarase (EC 3.2.1.81) digestion method of Finklestein & Rownd (1978).

Restriction endonuclease digestions. Cleavage of plasmid DNA with EcoRI (Boehringer) was carried out in 100 mM-Tris/HCl with 50 mM-NaCl and 10 mM-MgCl₂, pH 7.5, and with HindIII (Boehringer) in 10 mM-Tris/HCl with 50 mM-NaCl and 10 mM-MgCl₂, pH 7.6. Incubations were performed in 50 μl reaction mixtures containing 2–5 μg plasmid DNA and sufficient restriction enzyme to obtain complete digestion of the DNA in 2 h at 37 °C.

Electron microscopy of plasmid DNA. Purified plasmid preparations were employed, and spreading of DNA for electron microscopy was performed as described by Davis et al. (1971). When necessary, the plasmid DNA was converted to its oc form by incubation for 40 min at 37 °C with λ nuclease (EC 3.1.30.1; Sigma, from Aspergillus oryzae) at 10 units ml⁻¹ in 50 mM-sodium acetate plus 200 mM-NaCl and 1 mM-ZnSO₄, pH 4.5. Spread samples of plasmid DNA were 'stained' with 50 μM-uranyl acetate in 90% (v/v) ethanol and the preparations were examined using an AEI model 625B electron microscope. To obtain an estimate of the molecular size of a plasmid, its contour length was measured and compared with that of a standard plasmid DNA preparation, namely pBR322 in its oc DNA form (molecular mass 2.8 Md), placed on the same grid.

RESULTS AND DISCUSSION

When cleared lysates prepared from strains of C. butyricum were screened by agarose gel electrophoresis for the presence of plasmid DNA, plasmid(s) were easily detected in lysates from four strains (NCIB 7423, SA1, SA11, NCTC 6084) but were not found in the lysates of the remaining three strains (CNRZ528, CNRZ531, 855B). It was evident from the electrophoretic mobilities of the plasmid DNA bands in 0-8% and 1-4% agarose gels that all were relatively small plasmids of molecular mass less than 6 Md. It was conceivable that any large plasmid which may have been present in any of the strains of C. butyricum might have co-precipitated with the membrane and/or chromosomal DNA fractions in the course of the preparation of the cleared lysate (cf. Currier & Nester, 1976; Dobrista et al., 1978). Thus, as alternatives to the routine screening procedure (see Methods), two other preparatory methods were employed which should have ensured that any large plasmid would have been retained and included in the final DNA samples which were examined by agarose gel electrophoresis. These methods exploited alkaline denaturation either (a) of the cell lysates, as described by Hansen & Olsen (1978) or (b) of the pellet obtained by centrifugation of the cell lysates following addition of SDS (final concentration 4%, w/v) and 1 M-NaCl (Dobrista et al., 1978). In both cases the final DNA preparations were concentrated either by precipitation with ethanol (as in Methods) or with polyethylene glycol (PEG 6000; Merck) as described by Humphreys et al. (1975). No evidence of any large plasmid was obtained in any of the DNA preparations when these were eventually electrophoresed on 0-8% agarose gels.

Agarose gel electrophoresis of plasmid DNA from C. butyricum NCIB 7423

Purified preparations of plasmid DNA from C. butyricum NCIB 7423 migrated in four distinct bands on electrophoresis in 0-8% agarose gels (Fig. 1, track C). The two fastest running bands evidently contained the greater part of the total DNA. Limited digestion of the plasmid DNA with S₁ nuclease greatly diminished the intensities of these bands but increased the intensities of the two (original) bands of lesser mobilities. New bands of minor intensities were simultaneously generated which most probably consisted of linear fragments of DNA.

The DNA in each of the four bands obtained by agarose gel electrophoresis of the plasmid DNA preparation was retrieved by the agarase digestion procedure (see Methods). When these recovered DNA samples were once more subjected to electrophoresis in 0-8% agarose gels, the DNA preparations from the two slowest moving bands (cf. track C of Fig. 1) displayed their former mobilities, but the DNA preparations from the two originally faster moving bands displayed lesser mobilities which precisely corresponded to those of the former slower moving bands. It was concluded that the two bands of least mobility consisted of oc DNA and, by comparison with the oc DNA forms of the standard E. coli plasmids, it was
Fig. 1. Agarose gel electrophoresis of the purified plasmids of Clostridium butyricum strains NCIB 7423, NCTC 6084, SA1 and SA11. Also included on this 0.8% agarose gel were E. coli plasmids of known molecular sizes, namely pBR322, pHV14 and the plasmids of E. coli V517. Track A, plasmids of E. coli V517 (see below); B, pHV14; C, plasmids of C. butyricum NCIB 7423 (pCB101 and pCB102); D, plasmid of C. butyricum NCTC 6084 (pCB105); E, plasmid of C. butyricum strain SA1 (pCB103); F, plasmid of C. butyricum strain SA11 (pCB104); G, pBR322.

The bands of track A are: a, pVA517H (ccc DNA); b, pVA517G (ccc DNA); c, pVA517F (ccc DNA); d, pVA517H (cc DNA); e, pVA517E (ccc DNA); f, pVA517G (cc DNA); g, pVA517D (ccc DNA); h, pVA517C (ccc DNA); i, pVA517B (ccc DNA) and pVA517E (cc DNA); j, pVA517D (cc DNA); k, pVA517C (cc DNA); l, pVA517B (cc DNA). The molecular masses of plasmids pVA517H–pVA517B are 1.4, 1.8, 2.0, 2.6, 3.4, 3.7 and 4.8 Md, respectively.

estimated that they possessed molecular masses of 3.95 ± 0.19 Md and 5.56 ± 0.31 Md. The two faster moving DNA bands consisted of the covalently closed circular (ccc DNA) forms of what were evidently these two different plasmids which were designated as pCB101 and pCB102, respectively.

Agarose gel electrophoresis of plasmid DNA from C. butyricum strains SA1, SA11 and NCTC 6084

Purified plasmid DNA from each of these C. butyricum strains yielded two major DNA bands on electrophoresis in 0.8% agarose gels (Fig. 1, tracks D–F). A minor third DNA band yielded by the plasmid DNA preparation from strain SA11 was identified as the linear DNA form of the plasmid. Treatment of all three plasmid DNA preparations with S, nuclease in each case caused a substantial decrease in intensity of the fastest moving major DNA band (in 0.8% agarose gels) with a concomitant increase in intensity of the second major DNA band of lesser mobility. It was concluded that each of the plasmid DNA preparations contained a single plasmid present in both ccc DNA and cc DNA forms and, in the case of
Plasmids of Clostridium butyricum

Table 1. Molecular size determinations of plasmids by agarose gel electrophoresis and contour length measurements

<table>
<thead>
<tr>
<th>Host bacterium</th>
<th>Plasmid(s)</th>
<th>Molecular mass ± standard deviation (Md)</th>
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<tr>
<td></td>
<td></td>
<td>By agarose gel electrophoresis*</td>
</tr>
<tr>
<td>C. butyricum NCIB 7423</td>
<td>pCB101</td>
<td>3.95 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>pCB102</td>
<td>5.56 ± 0.31</td>
</tr>
<tr>
<td>C. butyricum SA1</td>
<td>pCB103</td>
<td>4.42 ± 0.29</td>
</tr>
<tr>
<td>C. butyricum SA11</td>
<td>pCB104</td>
<td>4.35 ± 0.11</td>
</tr>
<tr>
<td>C. butyricum NCTC 6084</td>
<td>pCB105</td>
<td>4.38 ± 0.08</td>
</tr>
<tr>
<td>E. coli AB2463</td>
<td>pHV14</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>E. coli HB101</td>
<td>pBR322</td>
<td>2.82 ± 0.10</td>
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ND, Not determined.

* Average correlation coefficient, 0.994.
† Plasmid pBR322 was employed as the internal standard and its molecular mass was taken as 2.80 Md (Ehrlich, 1978).

the strain SA11 preparation, its linear DNA form also. The plasmids were initially designated as follows: pCB103 in strain SA1, pCB104 in strain SA11, and pCB105 in strain NCTC 6084. Yet these plasmids were indistinguishable in size, each having a molecular mass of approximately 4.38 Md as estimated from the (identical) mobilities of their oc DNA bands on electrophoresis in 0.8% agarose gels (Table 1).

Electron microscopic examination of the C. butyricum plasmids

The presence of two plasmids (pCB101 and pCB102) in C. butyricum NCIB 7423 and of a single plasmid (pCB103) in C. butyricum SA1 was confirmed by electron microscopy. Approximately 70% of plasmids pCB101 and pCB102 were present as twisted ccc DNA, the remainder being oc DNA molecules. Limited digestion with S, nuclease converted the bulk of the plasmid DNA to the oc form, thus confirming the conclusions drawn from the behaviour of the plasmid DNA preparation when electrophoresed in 0.8% agarose gels. In the plasmid pCB103 preparation the majority of the molecules were in the supercoiled configuration with approximately 10% of oc DNA molecules.

To determine the sizes of the plasmids, each was converted to its oc DNA form by treatment with S, nuclease and was then mixed with twice the quantity of plasmid pBR322 similarly converted to its oc form. The average contour length of this standard E. coli plasmid was determined on each grid and was taken as the unit length corresponding to a molecular DNA mass of 2.80 Md. The molecular mass of the accompanying C. butyricum plasmid was then calculated from its average contour length. As a further control the molecular mass of plasmid pHV14 was similarly estimated and compared with its reported value of 4.6 Md. The results (summarized in Table 1) confirmed the estimates of the molecular masses of the C. butyricum plasmids made on the basis of their electrophoretic mobilities.

Digestion of C. butyricum plasmids with restriction endonucleases

Digestion of C. butyricum plasmids with EcoRI and HindIII yielded DNA fragments which were separated by electrophoresis on gels composed of various concentrations of agarose (from 0.8% to 1.4%). Reference DNA fragments of known molecular masses generated by digestion of bacteriophage λ with the same endonucleases were co-electrophoresed alongside the restricted plasmid samples. This bacteriophage genome has five cleavage sites for EcoRI (Thomas & Davis, 1975) and six sites for HindIII (Murray & Murray, 1975). The reference plot of log relative mobility versus log molecular mass so obtained proved to be linear only for DNA fragments of mass less than 4 Md.
Digestion with EcoRI of plasmids pCB103, pCB104 and pCB105 in each case yielded a single linear DNA fragment. These fragments were indistinguishable on the basis of their electrophoretic mobility in agarose gels. Similarly, all three plasmids yielded four DNA fragments on restriction with HindIII. Again, corresponding fragments from the three plasmids were apparently of identical size, with estimated molecular masses of 1·7, 1·03, 0·74 and 0·69 Md. It was, therefore, concluded that plasmids pCB103, pCB104 and pCB105 are probably identical, i.e. C. butyricum strains SA1, SA11 and NCTC 6084 carry a single common plasmid. Analysis of the products of digestion by a variety of other restriction endonucleases of the plasmid DNA preparations from these three strains, supplemented with heteroduplex analysis, should however be undertaken to verify this conclusion.

Plasmids pCB101 and pCB102 carried by C. butyricum NCIB 7423 were of different sizes and behaved differently when treated with enzymes EcoRI and HindIII. Plasmid pCB101 (3·9 Md) was not cleaved by EcoRI while plasmid pCB102 (5·2 Md) yielded two DNA fragments; the larger was of 4·5 Md. A mixture of pCB101 and pCB102 after treatment with HindIII yielded two major DNA bands when electrophoresed on 0·8% agarose gels. One of these consisted of DNA of 2·2 Md but the second DNA band was so diffuse as to suggest that it was not homogeneous. Accordingly, the HindIII-restricted DNA preparation was further electrophoresed in several gels containing agarose at concentrations from 0·6% to 1·4%. By this means two additional fragments (of 2·95 and 3·50 Md) were separated. Since neither pCB101 nor pCB102 was individually treated with HindIII it was not possible to conclude how many HindIII restriction sites were present in each plasmid. It was sufficient for our present purpose that although each plasmid was susceptible to digestion by HindIII neither was restricted in the manner of pCB103.

It is reasonable to suppose that either pCB101 or pCB102 specifies butyricin production by the butyricinogenic C. butyricum NCIB 7423. However, we have as yet been unsuccessful in attempts to select a clone of this strain which has lost either plasmid, even following exposure of the organism to various concentrations of several reagents which have provoked loss of plasmid-encoded functions from other bacteria. Plasmid pCB103 is similarly cryptic. Whilst plasmid-bearing strains of C. butyricum are evidently common, none of many strains of C. pasteurianum which we have examined was found to harbour a plasmid (N. Minton & J. G. Morris, unpublished observations).

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REFERENCES


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