

CHARACTERIZATION OF CHROMOSOMAL AND MEMBRANE ASSOCIATED PLASMID IN *BACILLUS BREVIS* ATCC 9999

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A covalently closed circular (ccc) DNA, with a weight of 44.7×10^6 daltons, has been isolated from *Bacillus brevis* ATCC 9999 (a gramicidin S producer) and from the gramicidin S-negative mutant EB16. The ccc DNA in the case of the parent strain, is mainly (99%) attached to the chromosomal and membrane fraction. A restriction enzyme map of the plasmid DNA was constructed for the enzymes *SalI*, *SmaI* and *BamHI*, which cleaved the plasmid DNA into two, two and six fragments respectively. Further digestion with the endonucleases *EcoRI* and *HindIII* cleaved the plasmid into 17 and 22 fragments.

Plasmids, or extrachromosomal DNA, have been identified in many bacterial species^{1,2,3}. There have been previous reports in *Streptomyces* about the involvement of extrachromosomal elements in various biochemical functions, such as antibiotic production⁴, fertility character⁵ and antibiotic resistance⁶. However, most of the characterised plasmids in bacilli^{7,8}, are cryptic elements and lack any known genetic information.

Previous studies in this laboratory have shown that gramicidin S-negative mutants appear with high frequency after treatment of the parent strain with ethidium bromide or acridine orange⁹. As a satisfactory explanation for these results, we believe that extrachromosomal DNA may play some role in antibiotic production, or, in the regulation of gene(s) expression in the producer strain.

In this paper, we describe the isolation of a totally membrane- or chromosome-associated plasmid from the gramicidin S producer *B. brevis* ATCC 9999 and the gramicidin S-negative mutant EB16. A restriction endonuclease cleavage map has been constructed.

Materials and Methods

Organisms and Media

Bacillus brevis ATCC 9999 was obtained from the American Type Culture Collection, and the gramicidin S-negative mutants were derived from it. Media and growth conditions were performed as described⁹.

Antibiotic Resistance

Resistance to kanamycin, streptomycin, lincomycin, nalidixic acid, colistin sulfate, trimethoprim and sulphafurazole were tested with filter discs containing these antibiotics. (Concentrations are indicated in results.)

Isolation of the Plasmid DNA

For the rapid isolation of plasmid DNA, the alkaline denaturation method of CURRIER and NESTER¹⁰, with modifications, was used. Overnight cultures were diluted 1/10 with nutrient broth, and the growth was continued in an 8-litre fermenter at 37°C for 6 hours. The cultures were harvested by centrifugation and washed twice with TE buffer containing 0.05 M tris (hydroxymethyl) amino-methane and 0.02 M ethylenediaminetetraacetate (EDTA), pH 8.0. Cells (20 g) were suspended in

250 ml TE buffer and a predigested pronase B (1 hour/37°C) was added to a concentration of 500 µg/ml. The suspension was incubated for 5 minutes at 37°C. Sodium dodecyl sulfate (20% in TE buffer) was then added to a final concentration of 1.5%. Complete lysis was achieved after 20 minutes incubation at 37°C. The lysate was mixed at 300 rpm for 2 minutes and the pH was adjusted to 12.3 by dropwise addition of 3 N NaOH under stirring. After 10 minutes stirring (300 rpm), the pH was reduced to pH 8.5 by dropwise addition of 2 M tris-HCl pH 6.0. The lysate was adjusted to 3% (w/v) NaCl and an equal volume of phenol (saturated in 3% NaCl) was added. The mixture was stirred for 5 minutes and the aqueous phase was separated from the phenol phase by centrifugation. The residual phenol was removed from the aqueous phase by extraction with an equal volume of chloroform - isoamyl alcohol (24: 1). To precipitate the DNA, 0.3 volume of 3 M sodium acetate pH 6.0 and an equal volume of ice-cold absolute ethanol were added. After standing overnight at -20°C, the precipitated DNA was collected by 10 minutes centrifugation (5000 rpm) and dissolved in minimum volume of 0.1 M EDTA, pH 8.0. The concentrated DNA was dialysed against TES buffer (30 mM tris-HCl, 5 mM EDTA and 50 mM NaCl, pH 8.0) overnight. To the dialysed DNA solution, 1 g/ml of CsCl and 0.1 mg/ml of ethidium bromide (10 mg/ml) were added. The DNA solutions were centrifuged at 42,000 rpm and 15°C for 44 hours using a 50 Ti rotor (Centrifuge L-5-65, Beckmann). The presence of plasmid DNA band was located with a long-wavelength UV light source. Ethidium bromide was extracted from the DNA with isoamyl alcohol and CsCl was removed by exhaustive dialysis against TES buffer. The plasmid DNA was concentrated by ethanol precipitation as described above.

For detection of plasmid DNA in the supernatant and the rapidly sedimenting fractions, the cell lysate was fractionated by centrifugation at 18,000 rpm for 30 minutes. From both fractions plasmid DNAs were isolated as described above.

Restriction Enzyme Digestion and Agarose Gel Electrophoresis

Digestion of the plasmid DNA was performed in a buffer containing 10 mM tris-HCl, pH 7.5, 10 mM MgCl₂ and 50 mM NaCl for *EcoRI* and *SalI*. The same buffer containing 5 mM 2-mercaptoethanol was used for *BamHI* and *HindIII*. *SmaI* digestion buffer contained 20 mM tris-HCl, pH 9.0, 15 mM KCl and 10 mM MgCl₂.

For complete digestion, 0.5~1 µg DNA was added to 1~2 µl of the corresponding restriction endonuclease. The digested DNA was analysed in a 0.7% agarose slab gel in 0.04 M tris-acetate buffer, pH 8.0, containing 0.02 M sodium acetate and 0.001 M EDTA, as described¹¹.

The restriction fragments were visualized by staining in aqueous ethidium bromide (1 µg/ml) solution for 30 minutes, and exposure to UV light (254 nm). *HindIII* and *EcoRI* digested λ DNA was used as molecular weight reference¹².

Electron Microscopy

The preparation of the plasmid DNA for electron microscopy was performed by spreading 10 µl of a solution containing 0.5 M ammonium acetate, 5 mM tris-HCl, pH 8.5, 1 mM EDTA, 0.01% cytochrome C and 2 mg DNA/ml on a hypophase of 0.25 M ammonium acetate¹³. Photographs were taken with a Philips EM 301 at primary magnification of 7,200.

Reagents

The restriction enzymes *SalI*, *SmaI*, *BamHI*, *HindIII* and *EcoRI* were from Boehringer-Mannheim, as well as pronase B. All antibiotics used were from Oxoid.

Results

Isolation and Characterisation of the ccc DNA

The normal procedures for preparing clear lysates currently used in *E. coli*, were not effective in detecting extrachromosomal DNA in *B. brevis*. Only when the supernatant was highly concentrated, were traces of plasmid DNA detected (Fig. 1). It has been reported that some plasmids co-precipitate with the chromosomal and membrane fraction^{11,14}. For detection of plasmid DNA in *B. brevis*, the

rapidly sedimenting fraction was subjected to the alkaline denaturation method¹⁰. With this method, we were able to detect a heavy band of ccc DNA (150~200 μ g ccc DNA/20 g cells) in the pellet fraction (Fig. 1). Most of the plasmid DNA in the parent strain was found to be attached to the chromosomal and membrane fraction, and only in the case of EB16 mutant, was a small amount (5~10%) of the ccc DNA detected in a free state. This may be due to the destruction of the association during the preparation, since the EB16 mutant has a different cell morphology to that of the wild type.

The size of the intact DNA molecules isolated from the parent strain and EB16, was investigated by electron microscopy. The contour length measurements of the plasmid DNA from the two strains, revealed on difference in length. A mean molecular weight of $44.7 \pm 0.7 \times 10^6$ was calculated using pBR322 as an internal standard (Fig. 2).

The cleavage patterns of the two plasmids were indistinguishable in molecular weight and in the set of fragments when cleaved by the endonucleases *SalI*, *SmaI* and *BamHI*. For further characterisation of the ccc DNA, the wild type plasmid was mainly used.

Fig. 2. Electron micrograph of *B. brevis* plasmid DNA (ccc and oc) and the distribution of contour lengths of open circular (oc) DNA molecules.

The small molecules are pBR322 dimers.

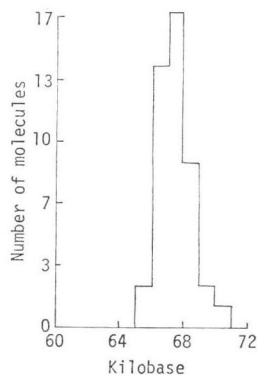
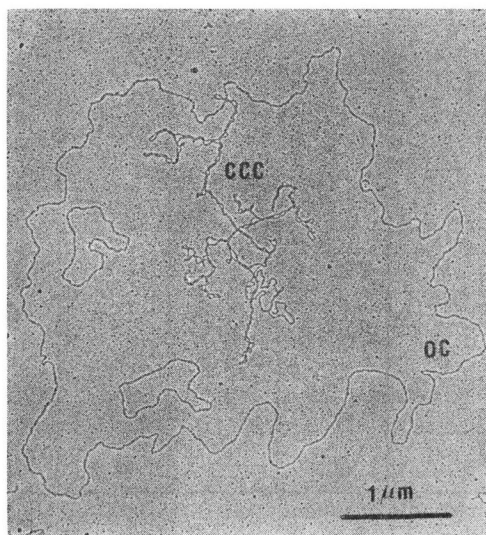


Fig. 1. CsCl-ethidium bromide density gradient centrifugation (parent strain).

(a) DNA from pellet fraction. (b) DNA from concentrated cell lysate supernatant.

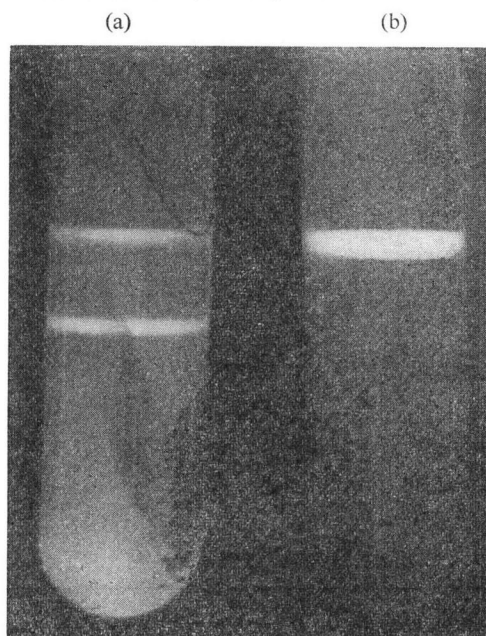


Fig. 3. Agarose gel electrophoresis of the ccc DNA cleaved with the endonucleases. 1, *SalI*; 2, *SalI+SmaI*; 3, *SmaI*; 4, *EcoRI* and 5, *HindIII*. Marker: 6, *EcoRI* fragments of λ DNA.

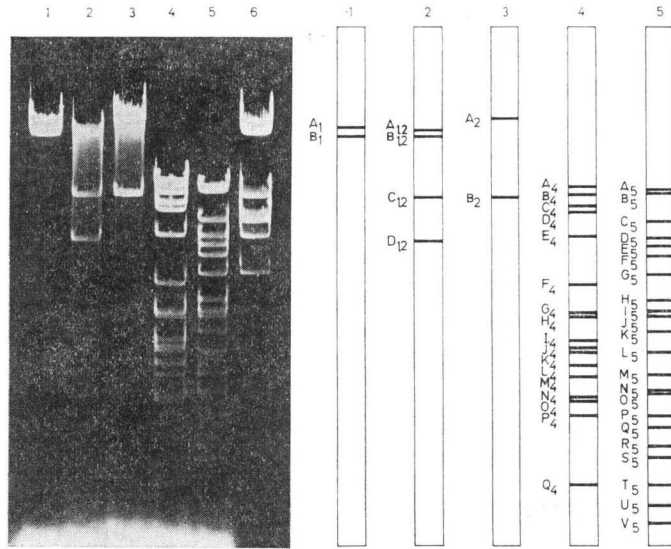


Table 1. Molecular weights ($\times 10^6$) of ccc DNA fragments after endonuclease digestion.

Fragment	Restriction enzyme							
	<i>SalI</i>	<i>SalI SmaI</i>	<i>SmaI</i>	<i>SmaI BamHI</i>	<i>BamHI</i>	<i>BamHI SalI</i>	<i>EcoRI</i>	<i>HindIII</i>
A	26.6*	18.7	40*	16.2	22.1*	12.3	5.6	5.35
B	18.3	18.3	4.9	10.1	10.1	9.8	5.3	5.2
C		4.9		8.3	8.3	8.3	4.5	3.6
D		3.0		4.9	2.2	6.8	4.2	3.0
E				2.2	1.3	3.3	3.1	2.8
F				1.3	0.9	2.2	1.8	2.5
G				1.0		1.3	1.6	2.1
H				0.9		0.9	1.51	1.82
I							1.20	1.63
J							1.15	1.52
K							1.10	1.45
L							0.95	1.12
M							0.85	0.98
N							0.72	0.93
O							0.65	0.73
P							0.55	0.52
Q							0.25	0.48
R								0.35
S								0.30
T								0.25
U								0.23
V								0.21
Σ		44.9		44.9		44.9	35.03	37.07

* Molecular weights calculated from double digestion.

Σ Is the sum of molecular weights.

Endonuclease Digestion

The restriction enzyme *SalI* cleaves the plasmid DNA into two fragments: A1 (26×10^6 daltons) and B1 (18.3×10^6 daltons). The restriction enzyme *SmaI* also gives two fragments: A2 (40×10^6 daltons) and B2 (4.9×10^6 daltons). A *SalI* and *SmaI* double digestion of the ccc DNA yields four fragments: A1,2, B1,2, C1,2 and D1,2 of approximate molecular weights of 18.7×10^6 , 18.3×10^6 , 4.9×10^6 and 3.0×10^6 respectively (Fig. 3). Fragment B1 is identical to B1,2 and B2 is also the same as C1,2. In the double digestion, both cut sites of *SmaI* are inside *SalI* fragment A1, whereas three fragments are produced next to the uncut fragment B1. The accurate molecular weights of the fragments A1 and A2 were calculated from the double digestion with *BamHI* (Table 1). Further digestion of the ccc DNA with *EcoRI* and *HindIII* produced 17 (A4-Q4) and 22 (A5-V5) fragments respectively (Fig. 3).

Complete cleavage of the plasmid DNA with the restriction enzyme *BamHI* produces six fragments: A3, B3, C3, D3, E3 and F3 with molecular weights of 22.1×10^6 , 10.1×10^6 , 8.3×10^6 , 2.2×10^6 , 1.3×10^6 and 0.9×10^6 (Fig. 5). The partial digestion of the plasmid DNA with *BamHI* (Fig. 4) produces five additional fragments next to the six main fragments determined on the basis of their mobility in gel electrophoresis, allows the construction of the following order for *BamHI* fragments: B3-D3-F3-C3-E3 and the largest *BamHI* fragment A3 closes this sequence into a circle.

Mapping of the Restriction Sites of *SalI*, *SmaI* and *BamHI*

The cleavage map with the relative positions of *SalI*, *SmaI* and *BamHI* was determined by gel electrophoresis of the fragments produced by two endonucleases. Double digestion of the ccc DNA with *SalI* and *BamHI* revealed eight fragments: A1,3~H1,3 with molecular weights between 12.3×10^6 to 0.9×10^6 . Among these fragments are C1,3, F1,3, G1,3 and H1,3 which are identical to the *BamHI* fragments C3, D3, E3 and F3 respectively (Fig. 5). Both restriction sites of *SalI* lie within *BamHI* fragments A3 and B3, and cleave them into four fragments. The *BamHI* fragment B3 (10.1×10^6 daltons) was cut by *SalI* to D1,3 (6.8×10^6 daltons) and E1,3 (3.3×10^6 daltons) and accordingly, A1,3 (12.3×10^6 daltons) and B1,3 (9.8×10^6 daltons) are the cleavage products of the *BamHI* fragment A3 (22.1×10^6 daltons).

Double digestion of the plasmid DNA with *SmaI* and *BamHI* also yields eight different fragments: A2,3~H2,3 with molecular weights between 16.2×10^6 to 0.9×10^6 (Fig. 5). The fragments B2,3, C2,3, E2,3, F2,3 and H2,3 are identical to the *BamHI* fragments B3, C3, D3, E3 and F3. Also the double digestion fragment D2,3 is identical to *SmaI* fragment B2. This double digestion shows that the two cut sites of *SmaI* are inside the *BamHI* fragment A3, which was cut into three fragments: A2,3

Fig. 4. Agarose gel electrophoresis of *BamHI* partial digestion.

2, 2 min; 3, 3 min; 4, 7 min; 5, 12 min; 6, 20 min. Marker: 1, *HindIII* fragments of λ DNA.

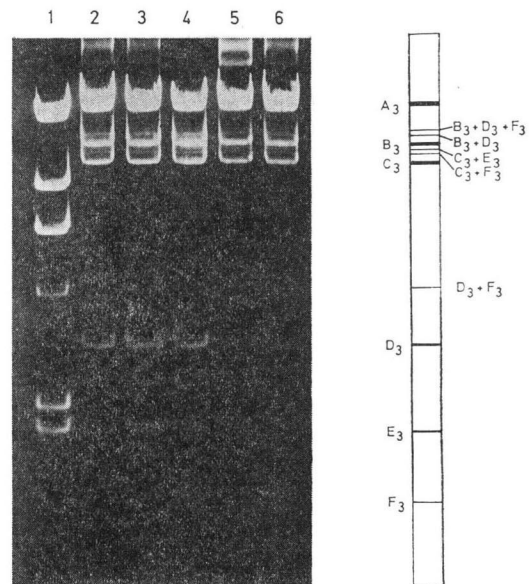
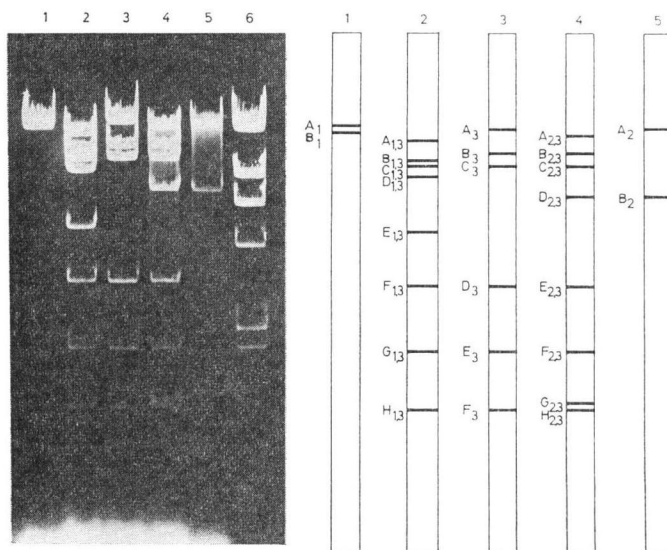


Fig. 5. Agarose gel electrophoresis of the ccc DNA cleaved with the endonucleases. 1, *SalI*; 2, *SalI*+*BamHI*; 3, *BamHI*; 4, *BamHI*+*SmaI* and 5, *SmaI*. Marker: 6, *HindIII* fragments of λ DNA.

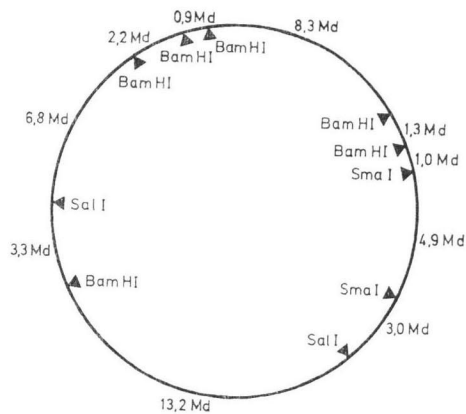


D2,3 and G2,3 with molecular weights of 16.2×10^6 , 4.9×10^6 and 1.0×10^6 . The restriction cleavage map with the relative positions of the restriction sites of *SalI*, *SmaI* and *BamHI* in the plasmid DNA is shown in Fig. 6.

Antibiotic Resistance

It is well known that plasmids often confer antibiotic resistance on their hosts. Looking for a possible function of the plasmid DNA, the resistance of wild type and EB16 was tested for several antibiotics. Both strains were found to be resistant to kanamycin ($30 \mu\text{g/ml}$), streptomycin ($25 \mu\text{g/ml}$), lincomycin ($2 \mu\text{g/ml}$), nalidixic acid ($30 \mu\text{g/ml}$), colistin sulfate ($10 \mu\text{g/ml}$), trimethoprim ($25 \mu\text{g/ml}$), and sulphafurazole ($500 \mu\text{g/ml}$).

Fig. 6. Cleavage map of the ccc DNA for the endonucleases *SalI*, *SmaI* and *BamHI*.



Discussion

The plasmid DNA from *B. brevis* is mainly associated with the chromosomal and membrane fraction. Only a small amount of the plasmid DNA ($5 \sim 10\%$) was detected in the clear lysate of EB16 mutant, and about $1 \sim 2\%$ of the total ccc DNA in the parent strain was found in a free state. In the case of the mutant, this may be due to the different cell morphology, or to the destruction of the association during preparation. Similar association of plasmid DNA with the rapidly sedimenting fraction in different bacterial species^{11,14,15} has been reported.

The contour length measurement of plasmid DNA revealed a mean molecular weight of 44.7×10^6 , which agreed with sums of the molecular weights calculated after the double digestion with *SalI*+*BamHI*.

*Sma*I, *Sma*I+*Bam*HI and *Sal*I+*Bam*HI. On the other hand, the sums of the molecular weights of *Eco*RI and *Hind*III fragments are noticeably lower than the value of the plasmid's molecular weight, which was calculated from its contour length. It is possible that some of the *Eco*RI and *Hind*III bands in gels may contain more than one DNA fragment.

The restriction cleavage map of the plasmid DNA for the endonucleases *Sal*I, *Sma*I and *Bam*HI was constructed on the basis of double and partial digestion of these endonucleases. The plasmid DNA contains two restriction sites for *Sal*I and *Sma*I, and six restriction sites for *Bam*HI.

The ccc DNA isolated from *B. brevis* ATCC 9999 appears very similar to the PAD1 plasmid (47.1×10^6 daltons) isolated from *B. brevis* var G.B.¹¹⁾. The two plasmids are membrane-associated and have the same cleavage sites for the endonucleases *Sal*I, *Sma*I and *Bam*HI. However, the double digestion of *B. brevis* ATCC 9999 plasmid DNA *Sma*I+*Bam*HI produced eight different fragments, whereas the PAD1 plasmid revealed only seven. Also the *Eco*RI and *Hind*III digestion of the PAD1 plasmid produced only 16 and 21 fragments respectively. The *B. brevis* var G.B. is a producer of gramicidin S and other antibiotics¹⁶⁾. This strain dissociates easily into four morphologically different variants¹⁷⁾ (R, S, P⁺, P⁻). On the other hand, the *B. brevis* ATCC 9999 did not dissociate and produces only gramicidin S.

To determine the possible function of the isolated plasmid, the resistance of wild type and EB16 to several antibiotics was tested. Both strains were found to be resistant to the aminoglycoside antibiotics—streptomycin and kanamycin, as well as to lincomycin, nalidixic acid, colistin sulfate, trimethoprim and sulphofurazole. Seventeen gramicidin S-negative mutants, isolated after treatment with the curing agents ethidium bromide or acridine orange, were screened for the ccc DNA (not shown). All these mutants were found to be plasmid-harboring, and to have the same resistance to the tested antibiotics as the parent strain. No direct association of the *B. brevis* plasmid DNA with a phenotypic trait has been detected, but further investigations are needed to elucidate its possible function.

To elucidate a possible function of the ccc DNA in sporulation, hybridization studies with total RNA isolated from the different growth phases of *B. brevis*, are under investigation.

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