Isolation and partial characterization of a new linear DNA plasmid isolated from *Bacillus polymyxa* SCE2

A. ROSADO and L. SELDIN*

Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, CCS, Bloco I, Ilha do Fundão, Rio de Janeiro, RJ, 21944-970, Brazil

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A plasmid, designated pFS1, has been identified for the first time in a strain of *Bacillus polymyxa*. The plasmid is 16 kb in length. It is sensitive to digestion by DNAase I, restriction endonucleases and exonuclease III, but resistant to RNAase A and λ -exonuclease. A restriction map of the plasmid has been constructed, demonstrating its linear nature. The linear DNA molecules of pFS1 are attached at their 5' ends to a protein which can be cleaved by Pronase. No homology was detected between pFS1 and chromosomal DNAs from a number of different *B. polymyxa* strains. Also, hybridization between pFS1 and total DNA from *B. polymyxa* SCE2 plasmid-cured derivatives does not occur. The plasmid seems to be cryptic since *B. polymyxa* strains harbouring pFS1 and the cured derivatives have the same phenotype.

Introduction

Linear DNA plasmids have been found in various yeasts (Gunge *et al.*, 1981; Gunge, 1983; Kitada & Hishinuma, 1987; Ligon *et al.*, 1989), fungi (Francou, 1981; Hashiba *et al.*, 1984; Meinhardt & Esser, 1984; Yui *et al.*, 1988; Kempken *et al.*, 1989; Kim *et al.*, 1990) and plants (Palmer *et al.*, 1983). The linear form is shared with various transposons (Grindley & Reed, 1985), adenoviruses (Tamanoi & Stillman, 1983) and the bacteriophage ϕ 29 from *Bacillus subtilis* (Escarmis & Salas, 1981). Among eubacteria, *Streptomyces* (Hayakawa *et al.*, 1979; Hirochika & Sakaguchi, 1982; Hirochika *et al.*, 1984; Kinashi *et al.*, 1987; Kinashi & Shimaji-Murayama, 1991), *Nocardia* (Kalkus *et al.*, 1990) and *Borrelia* (Barbour & Garon, 1987) seem to be the only genera having strains that harbour linear plasmids, whereas the great majority of plasmids described in other genera are found as covalently closed circular DNA molecules. Many *Bacillus* strains carry endogenous plasmids, several of which do not express useful or identifiable genetic markers (Lovett & Bramucci, 1975; Tanaka & Koshikawa, 1977; Tanaka & Sakaguchi, 1978; Uozumi *et al.*, 1980; Gryczan, 1982). A few of these plasmids have been used as cloning vectors for understanding certain biological phenomena. However, in *B. polymyxa*, no plasmids had previously been described.

Our interest in studying *B. polymyxa* genetics is due to the possible use of this bacterium in certain industries. Strains of this species produce antibiotics and enzymes that degrade various carbohydrates and polysaccharides (Debabov, 1982). Furthermore, some *B. polymyxa* strains are nitrogen-fixing (Seldin *et al.*, 1983; Claus & Berkeley, 1986). When looking for strains with genetic markers which could be used in recombination experiments, we isolated a *B. polymyxa* strain harbouring a plasmid, named pFS1. We have studied this plasmid using restriction endonucleases and exonucleases, and the results obtained suggest that it is linear and has a protein covalently associated with the 5' termini of the DNA molecules. pFS1 is therefore the first plasmid isolated from *B. polymyxa* and the first linear plasmid described in the genus *Bacillus*.

Methods

Bacterial strains and culture conditions. The strains used in this work are listed in Table 1. The bacteria were stored aerobically at room temperature on GB agar slants supplemented with 1% (w/v) CaCO₃ (Seldin *et al.*, 1983) and subcultures were made in GB broth (Seldin *et al.*, 1983) and incubated at 32 °C. Growth of a broth culture of *B. polymyxa* SCE2 with SDS (2 μ g ml⁻¹) was used to cure strain SCE2 of pFS1, as described previously (Bastos & Penido, 1981).

DNA extraction. Bacterial strains, including *B. polymyxa* SCE2, were grown in 100 ml GB broth. The cells were harvested and washed once with TE buffer (10 mM-Tris/HCl, pH 7.8, 1 mM-EDTA pH 7.5). The washed cells were resuspended in 10 ml of lysis buffer (Giambiagi-Marval *et al.*, 1990). Lysozyme (Calbiochem; 1 mg ml⁻¹) was added

* Author for correspondence. Tel. 21 270 8344; fax 21 259 9957.

Table 1. *Bacterial strains and plasmids*

Strain	Plasmid	Size (kb)	Phenotype associated with plasmid	Plasmid geometry	Source or reference
<i>Bacillus polymyxa</i> SCE2	pFS1	16	—	Linear	This work
42	—	—	—	—	pFS1 cured-derivative
43	—	—	—	—	pFS1 cured-derivative
Loutit*	—	—	—	—	Line & Loutit (1971)
DSM 372*	—	—	—	—	P. Jurtshuk, Jr, University of Houston, Texas, USA.
DSM 292*	—	—	—	—	
NRRL B-369	—	—	—	—	Laboratory of Microbiology, University of Delft, NL.
LMD24.16†	—	—	—	—	
LMD48.22†	—	—	—	—	Hayakawa <i>et al.</i> (1979)
<i>Streptomyces rochei</i> 7434-AN ₄	pSLA2	17	Lankacidin production	Linear	
<i>Staphylococcus aureus</i> MB41	pRJ22	15.4	Erythromycin resistance	c.c.c.	Hirochika & Sakaguchi (1982)
					M. C. F. Bastos, Institute of Microbiology, UFRJ, Rio de Janeiro, Brazil.

* Nitrogen-fixing strain.

† Polymyxin producer.

and the cells were incubated at 37 °C for 1 h. Then 500 µl of Pronase (Sigma; 10 mg ml⁻¹) was added and the preparation incubated at 37 °C for 1 h. Lysis was completed by addition of 2 ml SDS (10%, w/v). Following incubation at 37 °C for 30 min, the samples were heated at 65 °C for 15 min. Then KCl was added to 0.4 M and the samples were transferred to ice for at least 20 min. The lysates were cleared by centrifugation at 12000 *g* for 10 min. Nucleic acids were allowed to precipitate overnight with 2 vols ethanol at -20 °C and the DNA pellet was dissolved in TE buffer.

Isolation of linear plasmids pFS1 and pSLA2. Plasmid DNA was separated from total DNA by electrophoresis in 0.6% (w/v) agarose horizontal slab gels (Maniatis *et al.*, 1982). The band corresponding to the plasmid molecules was eluted from the gels as described by Thuring *et al.* (1975). Plasmid DNA was then purified by using a DEAE-cellulose (DE-52, Whatman) column (Schleif & Wensink, 1981).

Purification of plasmid pRJ22. Plasmid pRJ22 was purified by caesium chloride/ethidium bromide density-gradient centrifugation of cleared lysates prepared as described by Novick *et al.* (1979).

Enzymes. The restriction endonucleases were purchased from Boehringer Mannheim; λ-exonuclease and exonuclease III were from Gibco-BRL; all were used as specified by the manufacturer.

DNA radiolabelling, Southern transfer and hybridization conditions. DNA was blotted from gels to Millipore HAWP 345.05 nitrocellulose filters as described by Maniatis *et al.* (1982). Prehybridization and hybridization of filters were done as described previously (Seldin *et al.*, 1989). pFS1 was nick-translated as described by Davis *et al.* (1980). It was radiolabelled with α-[³²P]dATP to a level of about 5 × 10⁷ c.p.m. (µg DNA)⁻¹.

Results and Discussion

Physical characteristics of the plasmid

A *B. polymyxa* strain, named SCE2, was isolated from soil taken from Cerrado, Brasília, Brazil, and shown to

Fig. 1. Agarose gel electrophoresis of DNA from *B. polymyxa* SCE2. Lane A, RNAase-A-treated plasmid-containing preparation; lane B, DNAase-I-treated; lane C, untreated DNA preparation; lane D, pFS1 cured derivative strain 43; lane E, pFS1 cured derivative strain 46; lane F, *Hind*III fragments of bacteriophage λ DNA.

Table 2. Sizes of restriction fragments of plasmid pFS1

Fragments are in order of decreasing size.

Single and double digestions with:	Fragments (kb)					
<i>Sma</i> I	10	5.9				
<i>Sal</i> I	12.1	3.9				
<i>Ban</i> I	13.5	1.7	0.8			
<i>Pvu</i> II	12.5	2.6	1.4			
<i>Bcl</i> I	6.1	5.0	3.9	0.8		
<i>Sma</i> I/ <i>Sal</i> I	6.1(2 ×)	3.9				
<i>Sma</i> I/ <i>Ban</i> I	10	3.4	1.7	0.8		
<i>Sma</i> I/ <i>Pvu</i> II	6.1(2 ×)	2.6	1.4			
<i>Sma</i> I/ <i>Bcl</i> I	5.0	4.1	3.9	1.9	0.8	
<i>Sal</i> I/ <i>Ban</i> I	9.5	3.9	1.7	0.8		
<i>Sal</i> I/ <i>Pvu</i> II	12	2.5	1.4			
<i>Sal</i> I/ <i>Bcl</i> I	6.1	3.9	3.1	1.9	0.8	
<i>Ban</i> I/ <i>Pvu</i> II	9.3	2.6	1.7	1.4	0.8	
<i>Ban</i> I/ <i>Bcl</i> I	6.1	5.0	1.7	1.3	0.8(2 ×)	
<i>Pvu</i> II/ <i>Bcl</i> I	6.1	4.0	2.6	1.8	0.8	< 0.8

Fig. 2. Restriction map of pFS1. The recognition sites for *Bcl*I (B), *Sal*I (S), *Pvu*II (P), *Sma*I (M) and *Ban*I (A) are indicated. * The precise orientation of these two fragments is not known.

harbour plasmid-like DNA. No plasmids had been described for this *Bacillus* species, so we decided to characterize this new plasmid, designated pFS1. Attempts were made to purify the plasmid DNA by caesium chloride/ethidium bromide density-gradient centrifugation but always a single DNA band only was visualized. However, the presence of the plasmid in different DNA preparations was confirmed by electrophoresis in 0.6% (w/v) agarose gels (Fig. 1, lane C).

pFS1 was shown to be insensitive to RNAase A after 65 °C heat-treatment (Fig. 1, lane A), but it was sensitive to DNAase I (Fig. 1, lane B). Its molecular size was first estimated to be between 16 and 17 kb from its relative mobility in agarose gels when compared with size standard markers such as λ DNA *Hind*III fragments or the 17 kb *Streptomyces rochei* plasmid pSLA2 (Sakaguchi, 1990).

Restriction endonuclease digestion of pFS1

The restriction map of plasmid pFS1 was constructed after single and double digestions with *Sma*I, *Sal*I, *Ban*I, *Pvu*II and *Bcl*I. Table 2 shows the size of the fragments

Fig. 3. Agarose gel electrophoresis of untreated or Pronase-treated samples of plasmids pSLA2 (linear, lanes A and B), pFS1 (lanes C and D) and pRJ22 (c.c.c., lanes E and F). Lanes A, C and E contain cleared lysates prepared with Pronase treatment; lanes B, D and F contain cleared lysates prepared without Pronase treatment before phenol/chloroform purification.

generated by these endonucleases. The sum of the molecular weights of the restriction fragments was approximately equal to the size of the intact plasmid determined by comparison with size markers (Fig. 1). No restriction sites were detected for *Afl*II, *Asp*718, *Bgl*II, *Bst*BI, *Cla*I, *Eco*RI, *Kpn*I, *Mlu*I, *Nae*I, *Pml*I, *Sac*I, *Sac*II, *Sca*I, *Stu*I and *Xho*I. Various fragments were obtained when pFS1 was digested with *Acc*I, *Ava*I, *Bst*EII, *Bst*NI, *Dra*I, *Eco*RV, *Hae*II, *Hha*I, *Hind*III and *Hpa*I, but these enzymes were not chosen for further determination of the restriction map of pFS1.

Fig. 2 shows the restriction map of pFS1, which supports its linear nature. For example, concerning the number of fragments generated by each single digestion, six fragments would be expected when *Sal*I and *Bcl*I were used in double digestions, if the molecule were a covalently closed circle. However, only five fragments of pFS1 were detected in this case. To obtain this map, the agarose gels were blotted to nitrocellulose membranes and hybridized with pFS1, in order to enhance visualization of the smallest bands. Even then, it was not possible to determine the exact order of the two small *Ban*I fragments (0.8 and 1.7 kb).

Fig. 4. Agarose gel electrophoresis of pFS1 (lanes A, B and C), pSLA2 (lanes D, E and F), pRJ22 (lanes G, H and I) and phage λ DNA (lanes J, K and L), treated or not with exonucleases. Lanes A, D, G and J contain untreated preparations, lanes B, E, H and K contain DNA preparations treated with exonuclease III and lanes C, F, I and L contain DNA preparations treated with λ -exonuclease.

Association of a protein with the ends of the plasmid DNA

The presence of a protein at the ends of the pFS1 molecule was examined by reduction of the electrophoretic mobility of DNA in agarose gels, as previously observed for other linear extrachromosomal DNA elements (Hirochika & Sakaguchi, 1982; Ligon *et al.*, 1989; Kinashi & Shimaji-Murayama, 1991). Cleared lysates containing pFS1 or pSLA2 (linear plasmid used as a positive control) were prepared without Pronase treatment and were extracted with phenol/chloroform (1:1, v/v). No plasmid DNA band was detected in either preparation (Fig. 3, lanes B and D). The plasmid DNA band appeared when the preparations were treated with Pronase before extraction with phenol/chloroform, or even without this last extraction. These results suggest firm binding of a protein to pFS1 DNA, as occurs with pSLA2 (Hirochika & Sakaguchi, 1982). We further examined the location of the protein on pFS1 by testing

the susceptibility of the plasmid to 3' and 5' exonucleases. As shown in Fig. 4, the pFS1-protein complex was sensitive to exonuclease III and resistant to λ -exonuclease. These results strongly suggest that the 3' end of the plasmid DNA is free and that the protein may be associated with the 5' end.

Currently, we have no data concerning the function of the terminal protein found in pFS1. Terminal proteins have been reported to be essential for DNA replication in phage ϕ 29 (Salas *et al.*, 1978), in adenoviruses (Sakaguchi, 1990), and in pSLA2 DNA from *Streptomyces* (Hirochika & Sakaguchi, 1982).

Lack of homology of pFS1 with B. polymyxa chromosomal DNAs

To determine if the plasmid DNA had homology to the chromosomal DNA from different *B. polymyxa* strains, the total DNA from six strains was digested with *Hind*III, electrophoresed in a 0.6% agarose gel, and

transferred to a nitrocellulose filter. The filter was probed with nick-translated pFS1 and no sequences homologous to the plasmid DNA were detected (not shown). This suggests that there is no homology between pFS1 and the structural and/or regulatory genes usually present on the chromosome of *B. polymyxa*.

Curing of plasmid DNA

B. polymyxa SCE2 was cured of plasmid DNA by exposure to SDS. The absence of pFS1 could be observed by agarose gel electrophoresis in two strains, 43 and 46 (Fig. 1, lanes D and E). To determine if the plasmid DNA had integrated into the chromosomal DNA of the plasmid-cured derivatives, total DNA from strains 43 and 46 was digested with *Hind*III, electrophoresed in a 0.6% agarose gel, and transferred to nitrocellulose. The filter was probed with nick-translated pFS1 under non-stringent conditions. Chromosomal DNA of the plasmid-cured strains shared no homology with plasmid DNA (not shown).

The two cured derivative strains were analysed with respect to their biochemical characteristics in order to find a phenotypic characteristic that could be encoded by pFS1. Eight physiological characteristics (relation to oxygen, reduction of nitrate, acetoin fermentation, starch, casein and cellulose hydrolysis, presence of catalase and gelatin degradation) were compared between the wild-type and the cured strains. The taxonomic characteristics of *B. polymyxa* SCE2 are presented elsewhere (Rosado & Seldin, 1993). Strains SCE2, 43 and 46 showed identical physiological properties and the same profile when submitted to API 50 CHB tests. Furthermore, both cured strains produced an antimicrobial substance active against yeasts, fungi, and different genera of Gram-positive and -negative bacteria, exactly as does the parental strain harbouring pFS1 (Rosado & Seldin, 1993). This indicates that none of the properties studied here can be related to the presence of plasmid pFS1 in *B. polymyxa* SCE2.

In other organisms, e.g. some fungi, linear plasmids may specify important phenotypes. The nuclear plasmids of *Kluyveromyces lactis* encode a killer system (Niwa *et al.*, 1981) and a linear plasmid from *Rhizoctonia solani* was found only in weakly pathogenic, slow-growing isolates (Hashiba *et al.*, 1984). In yeasts, linear plasmids have been shown to be the genetic basis for killer toxin production (Gunge *et al.*, 1981). In *Streptomyces*, linear DNA plasmids are involved in production of the antibiotic lankacidin (Hirochika & Sakaguchi, 1982).

pFS1 may be useful for the construction of a gene transfer vector, there being none currently available for *B. polymyxa*. As pFS1 is the first plasmid described for this *Bacillus* species, its characterization is an encourage-

ment for the development of genetic studies in this species. Attempts are being made to introduce a drug resistance gene into pFS1 to facilitate selection of plasmid-containing strains.

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