Biotechnology Letters **22:** 1273–1276, 2000.

Improved method for separation of high molecular weight DNA molecules from *Zymomonas mobilis*

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Received 12 May 2000; Revisions requested 17 May 2000; Revisions received 15 June 2000; Accepted 16 June 2000

Key words: migration conditions, *Zymomonas mobilis*

Abstract

An improved method for separation of large DNA molecules from *Zymomonas mobilis* was developed using a contour-clamped, homogeneous electric field technique. The preparation of DNA samples was optimized, as well as the migration conditions, to resolve the chromosomal DNA fractions obtained after digestion with *Sfi*I. A 22 s (45 h) pulse setting gave maximal separation of 240 to 109 kb fragments range, while 9 s (40 h), 3 s (35 h) and 1 s (30 h) settings produced the best separation of 120 to 64 kb, 64 to 34 kb fragments range and less than 35 kb fragments, respectively.

Introduction

Zymomonas mobilis is an obligatory fermentative, Gram-negative bacterium that utilizes glucose, fructose and sucrose by the Entner Doudoroff pathway leading to the production of ethanol and CO_2 (Swings & De Ley 1977). Because *Z. mobilis* is a highly interesting microorganism for its powerful activity of ethanol fermentation (Arcuri *et al.* 1980, Rodríguez & Callieri 1986, Rogers *et al.* 1982), this bacterium has been studied using molecular genetic and biochemical methods. *Z. mobilis* is a microorganism that possesses great importance from the technological point of view because the ethanol yield is similar to that of *Saccharomyces cerevisiae* and also because of its potential use in the production of heterologous proteins (Conway *et al.* 1987).

The technique of pulsed field gel electrophoresis (PFGE) allows separation of megabase-sized linear DNA fragments (Schwartz & Cantor 1984, Smith *et al.* 1988) and it is useful for the construction of bacterial chromosomes physical maps (Smith & Condemine 1990). Cloned genes may be easily located in these physical maps allowing the construction of genetic maps. The aim of this work was to determine the optimal conditions of CHEF for resolution of high molecular weight DNA fractions from *Z. mobilis*.

Materials and methods

Microorganism growth and DNA preparation

Zymomonas mobilis ATCC 29192 was grown on sucrose, 50 g l^{-1}; yeast extract, 10 g l^{-1}; $(NH_4)_2SO_4$ 1 g l^{-1}; $KHPO_4$ and $MgSO_4 \cdot 7H_2O$, 1 g l^{-1}, at 30 °C, until 1 mg ml^{-1} (dry weight). Chloramphenicol was then added at 180 μg ml^{-1} and incubation continued for a further 3 h (chloramphenicol is used to synchronize ongoing rounds of chromosomal replication and inhibit further rounds of replication) Cells were harvested by centrifugation, the pellet was washed with EDTA 0.1 M (pH 8.0) and resuspended in 0.2 ml of EDTA 0.1 M. Then, cell suspensions were mixed with 0.2 ml of 2% w/v agarose and dispensed into 0.1 ml plug molds. Agarose plugs were incubated at 37 °C for 24 h in lysozyme buffer (Tris-HCl 0.01 M pH 7.2, NaCl 0.05 M, sodium deoxycholate 0.2%, SDS 1% and lysozyme 2 mg ml^{-1}) with subsequent incubation at 50 °C 48 h in proteinase K buffer (EDTA 0.1 M pH 8, sodium deoxycholate 0.2%, SDS 1% and pro-

teinase K 2 mg ml^{-1}). Finally the plugs were washed with TE (Tris/HCl 0.01 M pH 8, EDTA 0.001 M pH 8) and PMSF (0.01 mM) to inactivate proteases and then stored in TE at 4 °C.

Restriction endonuclease

Restriction enzyme *Sfi*I was obtained from New England Biolabs. The following enzyme concentratrions were tested: 5, 10, 20 and 30 U/0.1 ml plug.

PFGE

Electrophoresis was performed with a CHEF-DR II apparatus (BioRad Laboratories). The electrophoresis was carried out in 0.5 × TBE (Tris/borate 0.045 M, EDTA 0.001 M) (Sambrook *et al.* 1989) at 12 °C. For the determination of the optimal concentration of agarose to be used in the preparation of gels, a gradient of agarose from 0.8 to 1.2% was tested. Gels were stained in 0.01% ethidium bromide and photographed under UV light. Mid Range I PFG DNA marker (New England Biolabs) was used as molecular size marker.

Results and discussion

According to the results obtained, the optimal conditions for separation of large *Z. mobilis* DNA molecules might be summarized as follows:

Cell concentration

Better resolution was obtained with the plugs A (7.5 × 10^7 cell) (Figure 1). Higher concentrations of cells (plugs B and C) increased the intensity of the bands, but the background was also more pronounced (Figure 1).

Concentration of agarose, lysozyme and proteinase K

According to the respective tests, 1% agarose was the optimal concentration for the preparation of gels, while the best results concerning the enzyme treatment of plugs were obtained using a mixture containing 2 mg ml^{-1} lysozyme and the same amount of proteinase K.

Restriction endonuclease digestion of plugs

The results showed (Figure 2) that 5 U/0.1 ml plug of *Sfi*I produced a partial digestion including some fragments larger than 209 kb. The digestion level increased

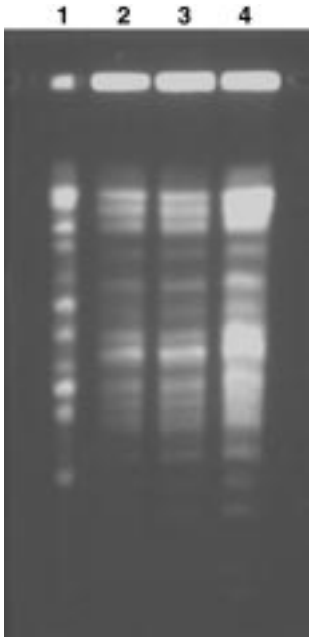

Fig. 1. CHEF. Relationship between the digestion pattern and the plug cell concentrations. Plugs were digested with 10 U/plug *Sfi*I. Lines: 1, PFGE Molecular Weight Marker Mid Range I; 2, plug A (7.5 × 10^7 cell); 3, plug B (2.2 × 10^8 cell); 4, plug C (9 × 10^8 cell). Running conditions: 3 V cm^{-1}, 9 s, 40 h at 12 °C.

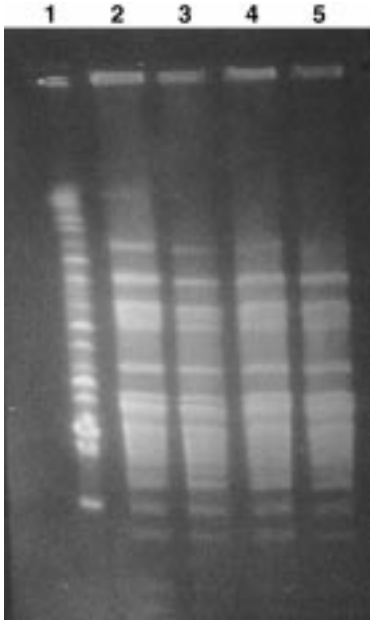

Fig. 2. CHEF. Relationship between the digestion pattern and the concentration of *Sfi*I. Plugs were digested with different *Sfi*I concentrations. Lines: 1, PFGE Molecular Weight Marker Mid Range I; 2, plug A digested with 5 U; 3, plug A digested with 10 U; 4, plug A digested with 20 U; 5, plug A digested with 30 U/100 μl of plug. Running conditions: 3 V cm^{-1}, 18 s, 48 h at 12 °C.

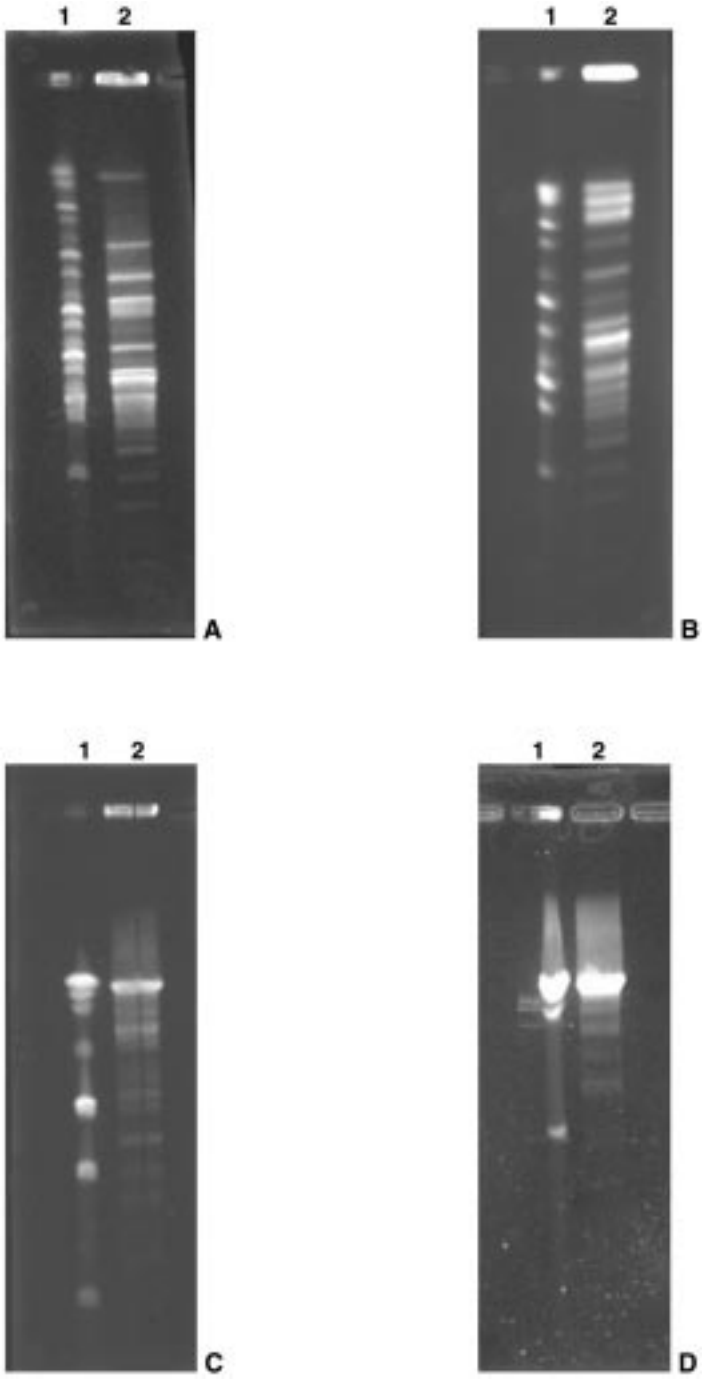

Fig. 3. CHEF. Different running conditions. Restriction fragments of *Z. mobilis* ATCC 29192 DNA. Lines: 1, PFGE Molecular Weight Marker Mid Range I; 2, Plug digested with *Sfi*I. A: 22 s pulse, 45 h run. B: 9 s pulse, 40 h run. C: 3 s pulse, 35 h run. D: 1 s pulse 30 h run. In all cases were used 3 V cm^{-1}.

Table 1. Running conditions: switch time and running time for the good resolution of those fragments.

Approximate fragments sizes	Switch time (s)	Running time (h)
33.5 kb – <15 kb	1	30
63.5 kb – 33.5 kb	3	35
112 kb – 63.5 kb	9	40
>209 kb – 112 kb	22	45

with 10 U/0.1 ml plug of *Sfi*I but no further effect was observed at higher enzyme concentration. Therefore, 10 U/0.1 ml plug of *Sfi*I was used to obtain the highest possible digestion under the experimental conditions used.

PFGE

To obtain reliable results when fragments of similar sizes are present, it is necessary to adjust the running conditions, i.e., voltage, migration time and switch time. Concerning this, Table 1 depicts the optimal conditions for the separation of four different ranges of fragment sizes. A 22 s (45 h) pulse setting gave a maximal separation of the largest fragments, between 240 to 109 kb, while 9 s (40 h), 3 s (35 h) and 1 s (30 h) settings gave the best separation of large (120 to 64 kb), medium (64 to 34 kb) and small (less than 35 kb) fragments, respectively (Figure 3).

Acknowledgements

The authors are indebted to CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina) for the support of this study. We thank to Mrs G.R.E. de Fanjul for the revision of the manuscript.

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