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AFLP fingerprinting of Colombian *Clostridium* spp strains, multivariate data analysis and its taxonomical implications

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Abstract

Amplified Fragment Length Polymorphism (AFLP) analysis was used for characterising 13 native Colombian *Clostridium* spp strains. The DNA extraction method was optimised and the use of cetyl trimethyl ammonium bromide (CTAB) and sodium chloride (NaCl) was incorporated. All strains could be typed in these conditions. The AFLP profiles obtained were submitted to multivariate analysis and compared with previous pulsed field gel electrophoresis (PFGE) results. The results suggested that the set of native strains could correspond to two new species different to those having been described to date. It is proposed that DNA–DNA hybridisation analysis should be done to produce complementary information for describing the new species.

Keywords: AFLP; PFGE; Bioprospecting; Fingerprinting; Taxonomic classification; Biodiversity

1. Introduction

Knowledge of prokaryote species' biological diversity remains incomplete in terms of their richness, overall distribution and function within Colombian ecosystems. Bioprospecting strategies have thus been implemented for exploring bacterial biodiversity, accompanied by molecular techniques for identifying promising microorganisms and their taxonomic classification.

Amplified fragment length polymorphism (AFLP) represents one of the molecular characterisation techniques used in taxonomic classification, based on detecting DNA restriction fragments for polymerase chain reaction (PCR) amplification (Zabeau and Vos, 1993). AFLP has been applied to studying different bacterial genera for both taxonomic ends and epidemiological typing, showing it to be a useful molecular tool because it allows genera, species and even strains to be differentiated (Blears et al., 1998; Gaafar et al., 2003; Moreno et al., 2003; Rademaker et al., 2003;Vos et al., 1995; Savelkoul et al., 1999). AFLP fingerprinting studies have been done recently on *Clostridium novyi*, *Clostridium perfringens*, *Clostridium botulinum* and

Abbreviations: AFLP, amplified fragment length polymorphism; PCR, polymerase chain reaction; OD, optical density; PFGE, pulsed field gel electrophoresis; CTAB, cetyl trimethyl ammonium bromide; DNA, deoxyribonucleic acid; NaCl, sodium chloride.

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Clostridium difficile (McLauchlin et al., 2002; Engström et al., 2003; Van den Berg et al., 2004; Keto-Timonen et al., 2005).

178 strains have been isolated from soil from different areas of Colombia in the search for solvent-producing microorganisms as part of the process of bioprospecting carried out in the country during the 1990s. Later analysis led to it being established that 13 of these isolated strains exhibited higher total solvent production than the *Clostridium acetobutylicum* ATCC 824 reference strain, using glucose as carbon source (Montoya et al., 2000b). Promising strains were characterised by PFGE fingerprinting (Montoya et al., 2001) and analysing the 16S rRNA gene sequence (Montoya et al., 2000a). The results suggested that native strains were homogeneous and that they were closely related to the *Clostridium butyricum* species. The *Hin*dIII enzyme was selected in preliminary AFLP genotyping assays but only 7 strains could be typed due to the quality of the DNA and the high endonuclease activity (data no shown). This work presents the results of AFLP fingerprinting for the 13 native strains, after DNA extraction methods had been optimised and multivariate analysis for establishing taxonomic relationships.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Strains IBUN 13A, IBUN 18A, IBUN 18Q, IBUN 18S, IBUN 22A, IBUN 62B, IBUN 62F, IBUN 64A, IBUN 95B, IBUN 125C, IBUN 137K, IBUN 140B, IBUN 158B were isolated from Colombian soils as described previously (Montoya et al., 2000b). Reference strains *C. acetobutylicum* ATCC 824, *C. butyricum* DSM 2478, *C. kainantoi* DSM 523, *C. beijerinckii* were kindly provided by Wolfgang Schwarz from Technical University of Munich. Reference strain *C. saccharoperbutylacetonicum N1-4* was kindly provided by D.J Jones. (University of Otago, New Zealand).

Conserving the microorganisms, activating the strains, anaerobic growth conditions and determining cellular biomass were carried out following the methodology described by Montoya et al., 2000b.

2.2. DNA extraction

A preinoculum was prepared by adding 1.5 ml of previously activated culture to 40 ml RCM medium in anaerobic conditions and incubated overnight at 37 °C. 7.5 ml of the preinoculum were transferred to flasks containing 100 ml RCM medium. It was grown at 37 °C until 0.3–0.5 OD and placed in an ice bath for 30 to 45 min. The cells were harvested by spinning at 5000 rpm for 10 min. 500 μl of the pellet were centrifuged at 12,000 rpm for 5 min and suspended in 567 μl TE buffer (10 mM Tris HCl, 1 mM EDTA). This was mixed by repeated pipetting using tips which had been cut shorter to widen them. SDS was added at final 0.5% concentration and proteinase K at final 100 μg/ml concentration. The mixture was incubated in a water bath at 37 °C for 1 h; 100 μl NaCl 5 M and 80 μl CTAB/NaCl solution were then added and completely mixed. After adding an equal volume of 24:1 chloroform:isoamyl alcohol mixture, it was incubated at 65 °C for 10 min and mixed for inversion until the two phases formed a homogeneous emulsion. It was centrifuged at 12,000 rpm for 10 min to remove the complex formed between CTAB-proteins and polysaccharides. Chloroform-isoamyl alcohol extraction was repeated three times until the aqueous phase became translucent. DNA was extracted from the aqueous phase with an equal volume of phenol/chloroform/isoamyl alcohol solution (25:24:1), mixed and centrifuged at 12,000 rpm for 10 min. DNase-free RNAse was added to the supernatant at final 20 μg/μl concentration and incubated at 37 °C for 30 min. The DNA was precipitated with two volumes of isopropanol and then recovered for centrifuging at 12,000 rpm for 20 min at 4 °C. The DNA pellet thus obtained was then washed with a volume of prechilled absolute ethanol and centrifuged at 12,000 rpm for 10 min. This step was repeated 3 times with prechilled 70% ethanol. The supernatant was skimmed off and the pellet was dried at 37 °C for 1 h. The pellet was suspended in 100 μl TE buffer and stored at −20 °C. DNA quality was verified by electrophoresis on 0.8% agarose gels.

2.3. AFLP fingerprinting

One μg DNA was digested with 10 U *Hin*dIII enzyme (Promega) in a final volume of 20 μl at 37 °C for 12 h. 20 μl of the digested DNA were transferred to a new tube containing 25 pmoles of adapters (5′ CTCGTAGACTGCGTACC 3′; 3′ CTGACGCATGGTCGA 5′) (Janssen et al., 1996) and 1 U DNA T4 Ligase, (Promega) in a final volume of 30 μl at 4 °C for 12 h. Enzymes were inactivated at 65 °C for 20 min after the digestion–ligation reaction had been finalised (Clerc et al., 1998). 50 ng digested–ligated DNA was amplified by PCR in 25 μl reaction volume with *Hin*dIII primer (5′ GACTGCGTACCAGCTT 3′ with an additional **C** at the 3′ extreme acting as the basis for selection) at 0.2 μM concentration, with 0.1 mM dNTPs, 1.25 U Taq polymerase (Promega).