Technical Note: A Rapid Pulsed-Field Gel Electrophoresis Method for Analysis of *Bifidobacteria*

E. P. Briczinski and R. F. Roberts¹

Department of Food Science, The Pennsylvania State University, University Park 16802

ABSTRACT

Pulsed-field gel electrophoresis (PFGE) is a widely used and highly discriminatory molecular typing method that has been applied to bifidobacteria. However, published PFGE protocols used with bifidobacteria require between 5 and 7 d to complete. A rapid PFGE method was developed that can be completed within 24 h.

Key words: pulsed-field gel electrophoresis, *Bifidobacterium*

As the amount of scientific evidence supporting the reported health benefits of probiotics has risen, interest has grown in adding probiotic organisms to dairy products and to pharmaceutical preparations. A number of selection criteria have been proposed for probiotic microorganisms and, in addition to the various functional, technological, and safety criteria, appropriate methods must exist to differentiate one strain from another (Mattila-Sandholm et al., 2002; Champagne et al., 2005). Nucleic acid-based techniques have become increasingly important in differentiating and typing bacteria and in providing a means for evaluating inter- and intraspecies relatedness (Busch and Nitschko, 1999). Pulsed-field gel electrophoresis (PFGE) is a widely used and highly discriminatory molecular typing method based on comparison of fragment patterns of restriction-digested chromosomal DNA (Basim and Basim, 2001). Various researchers have applied PFGE to *Bifidobacterium* strains to estimate chromosome size (Bourget et al., 1993), to assess differences within and between human fecal samples (McCartney et al., 1996; Kimura et al., 1997; Rosberg-Cody et al., 2004), and to differentiate strains (Roy et al., 1996; Simpson et al., 2003; Yeung et al., 2004). However, reported PFGE protocols used with bifidobacteria are not conducive to routine analysis, requiring between 5 and 7 d to complete the assay. The objective of this research was to

develop a PFGE protocol that could be applied to bifidobacteria and that could be completed within 24 h.

Twelve strains of *Bifidobacterium* were obtained from the ATCC (American Type Culture Collection, Manassas, VA) and the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), and 22 samples of *Bifidobacterium* were obtained from 6 commercial starter culture companies (Table 1). All strains were identified as *Bifidobacterium* by PCR amplification of a region of 16S rDNA based on the method of Kaufmann et al. (1997). Commercial strains were identified at the species level using PCR primers under the conditions described by Matsuki et al. (1999), Ventura et al. (2001), and Ventura and Zink (2002). Suspensions of strains from ATCC, DSMZ, and from commercial starter culture companies (4 strains: 2 *Bifidobacterium animalis* ssp. *lactis*, 1 *Bifidobacterium infantis*, and 1 *Bifidobacterium longum*) were prepared and separated into duplicate aliquots to compare the typical and modified protocols. All strains were then evaluated using the modified protocol. Comparisons of the typical and modified methods were replicated twice. The modified protocol was replicated with all strains at least in triplicate.

For PFGE, strains were streaked onto liver lactose agar (35 g/L liver infusion broth, 10 g/L lactose, 10 g/L trypticase peptone, 2 g/L sodium chloride, 15 g/L agar; Enumeration Procedures for Probiotics, Lyoferm, Inc., Indianapolis, IN) and incubated anaerobically for 3 d at 37°C in an anaerobic incubator (5 mmHg vacuum pressure; anaerobic mixed gas: 10% carbon dioxide, 5% hydrogen, 85% nitrogen; VWR, West Chester, PA). A single colony was transferred to 10 mL of liver lactose broth and incubated anaerobically overnight until turbid. A 2.0-mL aliquot was centrifuged for 10 min at 14,000 × *g* to pellet the cells, which were then washed once with 2.0 mL of 100 mM Tris, 100 mM EDTA buffer (pH 7.6) and resuspended in 600 µL of this buffer. This suspension was separated into duplicate 160-µL aliquots to compare the typical and modified PFGE protocols. To the 160 µL of cell suspension used with the typical protocol was added an additional 50 µL of the resuspension buffer to account for the dilution effect of

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¹Corresponding author: rfr3@psu.edu

Table 1. Strains of *Bifidobacterium* spp. evaluated in this study

Strain name ¹	Strain identity
ATCC 15696	<i>Bifidobacterium bifidum</i>
ATCC 15697	<i>Bifidobacterium infantis</i>
ATCC 15698	<i>Bifidobacterium breve</i>
ATCC 15700	<i>B. breve</i>
ATCC 15707	<i>Bifidobacterium longum</i>
ATCC 15708	<i>B. longum</i>
ATCC 25527	<i>Bifidobacterium animalis</i> ssp. <i>animalis</i>
ATCC 27536	<i>B. animalis</i> ssp. <i>lactis</i>
ATCC 27672	<i>B. animalis</i> ssp. <i>animalis</i>
ATCC 27686	<i>Bifidobacterium choerinum</i>
ATCC 29521	<i>B. bifidum</i>
DSMZ 10140	<i>B. animalis</i> ssp. <i>lactis</i>
	<i>B. animalis</i> ssp. <i>lactis</i> (20 commercial strains)
	<i>B. infantis</i> (1 commercial strain)
	<i>B. longum</i> (1 commercial strain)

¹ATCC = American Type Culture Collection (Manassas, VA); DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).

adding lysis reagents during plug preparation with the modified PFGE protocol.

The "typical" protocol selected was based on that described by Simpson et al. (2002, 2003), which is representative of the PFGE methods found in the bifidobacteria literature. Briefly, the cell suspension was mixed with an equal volume of 2% InCert agarose (Cambrex, Rockland, ME) prepared in 0.125 M EDTA (pH 7.6) and dispensed into disposable plug molds (10 × 5 × 1.5 mm; Bio-Rad, Hercules, CA). The plugs were incubated in 1 mL of 1 M NaCl, 6 mM Tris-HCl, 100 mM EDTA, 1% Sarkosyl buffer (pH 7.6; Sigma, St. Louis, MO) with 10 mg/mL lysozyme (Sigma) and 500 units/mL mutanolysin (Promega Corp., Madison, WI) at 37°C for 18 h. The plugs were then incubated in fresh Sarkosyl buffer with 0.8 mg/mL proteinase K (Sigma) at 37°C for 18 h. The proteinase K buffer was refreshed and the plugs were then incubated for an additional 18 h. The plugs were washed twice with 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) in 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) at 37°C for 60 min in a shaking water bath (New Brunswick Scientific, Edison, NJ). Two slices (1-mm wide) were prepared from the plugs and washed 3 times in 1 mL of 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0) for 15 min at room temperature. The slices were preincubated at 4°C for 30 min in 100 µL of the appropriate restriction endonuclease buffer. They were then transferred to 100 µL of a fresh restriction digest mixture containing 30 units of *Xba*I or *Spe*I and incubated at 37°C for 18 h.

To the cell suspension were added 40 µL of lysozyme solution (100 mg/mL) and 10 µL of proteinase K solution (20 mg/mL). This mixture was immediately combined with an equal volume of 1.6% InCert agarose prepared

in 0.1% SDS (International Biotechnologies, Inc., New Haven, CT) and dispensed into disposable plug molds. Lysis was performed by incubating the plugs in 1.5 mL of 0.5 M EDTA, 1% Sarkosyl buffer (pH 9.0) with 4 mg/mL lysozyme and 200 units/mL mutanolysin at 55°C for 90 min; the plugs were then incubated in fresh Sarkosyl buffer with 0.5 mg/mL proteinase K at 55°C for 60 min. The plugs were washed in preheated sterile distilled water at 50°C for 15 min, then in 3 changes of preheated 10 mM Tris, 1 mM EDTA buffer (pH 7.6) at 50°C for 15 min in a shaking water bath at 75 rpm. Two slices were prepared from the plugs and incubated in 100 µL of a restriction digest mixture with 30 units of *Xba*I or *Spe*I for 2 h at 37°C.

Electrophoresis was performed on 1.0% SeaKem Gold agarose gel (Cambrex; Michaud et al., 2001) using 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). Slices from the traditional and modified methods, from the same initial cell preparation, were loaded in adjacent lanes for comparison. A lambda ladder (Bio-Rad) was included as a molecular weight marker. Electrophoresis was performed using a CHEF Mapper System (Bio-Rad). Switch times were increased linearly from 0.19 to 35.38 s for 13.9 h, with an angle of 120° at 6 V/cm and 14°C. Gels were stained with a solution of ethidium bromide (0.4 mg/L; Promega) for 1 h, then destained for 2 h. Restriction patterns were visualized on a UV transilluminator (302 nm), and images were captured using an AlphaImager 3300 Gel Documentation System (Alpha Innotech Corp., San Leandro, CA) and saved as .TIFF files for future analysis.

Banding patterns obtained for each strain from both the typical and modified methods were identical (Figure 1), confirming the suitability of this rapid method with samples of bifidobacteria.

Several modifications to the typical protocol were responsible for reducing the time needed to perform this technique. Most of the reduction in time for this method was associated with shorter incubation times during lysis, washing, and restriction.

Bifidobacteria are typically more difficult to lyse than gram negative bacteria, and PFGE protocols with bifidobacteria generally use long lysis times (18 to 72 h). However, to reduce the lysis time, lysis reagents may be added to the initial cell suspension, then immediately combined with agarose to prevent undesirable shearing or degradation of the DNA, as recommended by Chang and Chui (1998). By adding lysozyme and proteinase K directly to the bifidobacteria cell suspension, incubation time in the lysis buffer with lysozyme and mutanolysin could be reduced to 90 min. A higher incubation temperature (55°C) was also used, which is closer to the temperature for maximum activity of the mutanolysin (Yokogawa et al., 1974). When the incubation time in this

Figure 1. Pulsed-field gel electrophoresis comparing the traditional and rapid protocols, digested with *Xba*I. M: Lambda molecular weight marker; lanes with “a” were performed with the traditional method, and lanes with “b” were performed with the rapid method; lanes 1a/b: *Bifidobacterium breve* ATCC 15698; lanes 2a/b: *B. breve* ATCC 15700; Lanes 3a/b: *Bifidobacterium longum* ATCC 15707; Lanes 4a/b: *B. longum* ATCC 15708; Lanes 5a/b: *Bifidobacterium animalis* ssp. *lactis* (commercial strain).

step was varied from 90 min to 18 h, no difference in the intensity of the bands was observed. Although many of the bifidobacteria strains lysed without the addition of mutanolysin, it was necessary to add mutanolysin (200 units/mL) to the buffer to ensure complete lysis of all 34 strains examined. In an effort to reduce reagent costs associated with the modified method, subsequent experiments using a lower concentration of mutanolysin (40 units/mL) were performed, which resulted in complete lysis of 33 of the 34 strains of bifidobacteria within 1.5 h. Therefore, this lower mutanolysin concentration of 40 units/mL has been used in our laboratory for routine PFGE analyses.

The incubation of the plugs in proteinase K buffer was also reduced from between 12 and 50 h, as in traditional bifidobacterial protocols, to 60 min in the rapid protocol. When this step was varied between 1 and 4 h, there was no difference in band intensity among the strains examined.

The washing steps were also modified from those of traditional PFGE protocols. Most protocols with bifidobacteria use PMSF, a serine protease inhibitor, to inactivate proteinase K, followed by a series of washes to remove the PMSF (McCartney et al., 1996; Roy et al., 1996; Sanders et al., 1996; Kimura et al., 1997; Crittenden et al., 2001; Ventura and Zink, 2002; Yeung et al., 2002; Gueimonde et al., 2004; Mättö et al., 2004). Some protocols have extensively washed plugs to remove the

proteinase K rather than inactivating it (Grand et al., 2003; O’Riordan and Fitzgerald, 1997). However, washing steps often will take up to 30 h in traditional methods. The rapid PFGE methods for *Escherichia coli* and *Campylobacter*, presented by Gautom (1997) and Michaud et al. (2001), respectively, suggested preheating the water or buffer and using a shaking water bath to shorten the time for this step. This procedure was applied in the rapid method for bifidobacteria—4 washes of 15 min each were performed with water and Tris, EDTA buffer at 50°C—without negatively affecting the subsequent restriction of the DNA.

Restriction was also shortened from a traditional overnight incubation to 2 h without increasing the amount of enzyme used. Other rapid methods have used digest times between 90 min and 3 h (Matushek et al., 1996; Gautom, 1997; Chang and Chui, 1998; Michaud et al., 2001). A long incubation time was not necessary with samples prepared according to the rapid method based on the identical patterns obtained when digestion occurred over 18 h.

A rapid PFGE method was developed for bifidobacteria that may be completed, from a turbid tube of culture media to a picture of a gel, within 24 h. Although no effort was made to determine the minimum incubation times necessary at each step, the times reported here have been used successfully with our collection of bifidobacteria strains, suggesting that the long times used in traditional methods are unnecessary. This method should allow PFGE to be more readily applied to bifidobacteria isolates in the dairy and supplement industries.

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