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NOTE

Pulsed-Field Gel Electrophoresis for the Identification of Bacteria Causing Milky Disease in Scarab Larvae

Paenibacillus popilliae and *Paenibacillus lentimorbus* are bacteria causing milky disease in several species of scarab larvae. The two bacterial species were shown to be separate by DNA hybridization studies (Rippere *et al.,* 1998; Harrison *et al.,* 2000). Although these studies delineated the species, it is not possible to recognize individual isolates by this technique. In clinical microbiology, the need to identify individual bacterial isolates as part of epidemiological studies tracing the movement of infectious agents between patients, staff, and areas of hospitals and on a larger scale during epidemics was approached by utilizing pulsed-field gel electrophoresis (PFGE) of DNA fragments produced by rare-cutting nucleases. The potential use of this technique in invertebrate pathology was reported by Macdonald and Kalmakoff (1995) who examined six New Zealand milky disease isolates and compared them to the North American type strain of *P. popilliae,* ATCC 14706. They found the New Zealand isolates to be easily distinguishable from ATCC 14706 and to be similar but distinguishable from each other. This result encouraged us to expand that study to a much larger number of milky disease isolates from different geographic areas and insect hosts.

The 19 North American, 19 Latin American, 2 European, and 1 New Guinean *P. popilliae* and *P. lentimorbus* isolates used in this study were those described by Rippere *et al.* (1998) and Harrison *et al.* (2000). The cells from 200 μl of broth were harvested by centrifugation and the pellet was suspended in 50 μl of cell resuspension buffer (10 mM Tris–HCl, pH 7.2, 50 mM sodium EDTA, 20 mM NaCl) and 2 μl of 25 mg/ml lysozyme. The cells were mixed with an equal volume of low-melting-point agarose and pipeted into a plug mold. The plugs were incubated for 1 h at 37°C with 0.5 ml of lysis buffer (10 mM Tris–HCl, pH 7.2, 50 mM NaCl, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine) and 15 μl of 25 mg/ml lysozyme. The lysis buffer was replaced with 0.75 ml of wash buffer (20 mM Tris, pH 8.0, 50 mM sodium EDTA). The plugs were incubated overnight at 50°C with a protein digestion buffer (100 mM sodium EDTA, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine) and 20 mg/ml proteinase K. Before digestion, the agarose plugs were washed four times for 1 h each time in 0.1× wash buffer at 22°C and for 1 h in 500 μl restriction buffer (NEB buffer 4) at 22°C. Digestion of genomic DNA was carried out by incubating the inserts at 4°C overnight and at 37°C for 2 h in 200 μl of fresh restriction buffer containing 2 μl of *Pme*I following the recommendations of the manufacturer (New England Biolabs). The chromosomal restriction fragments were analyzed by PFGE in a CHEF DRII system (Bio-Rad Laboratories) by loading small pieces of the plugs into slots of a 1% agarose gel prepared in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM sodium EDTA, pH 8.0). PFGE was performed in 2 L of 0.5× TBE equilibrated at 14°C for 20 h at 6 V/cm.

Of the 19 Latin American milky disease strains examined, strains 522 and 527 isolated from *Phyllophaga menetriesi* in Costa Rica showed identical PFGE band patterns. Also, strains 381 and 382 from the same insect species in Costa Rica yielded patterns identical to each other (Fig. 1). These two pairs of isolates gave very similar but easily distinguishable band patterns and are probably closely related. Four isolates from Costa Rica, 266 and 283 (both from *Phyllophaga*

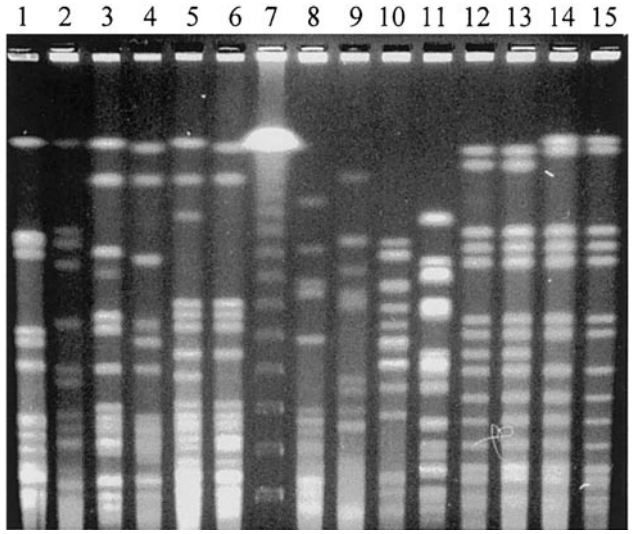

FIG. 1. All lanes are *P. popilliae,* Lanes 1, ATCC14706; 2, BpCh1; 3, DNG4; 4, DNG12; 5, DNG11; 6, A8; 7, ladder with sizes (kb) in left margin; 8, B2522; 9, 525; 10, 479; 11, 491; 12, 381; 13, 382; 14, 522; 15, 527.

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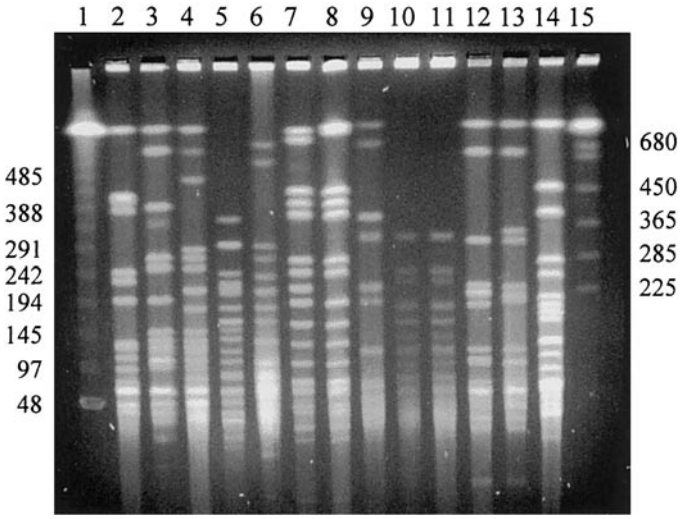

FIG. 2. Lanes 2–9 are *P. popilliae* and lanes 10–14 are *P. lentimorbus*. Lanes 1, ladder with sizes (kb) in left margin; 2, ATCC14706; 3, DNG4; 4, DNG11; 5, H1; 6, B4081; 7, 381; 8, 527; 9, 470; 10, 266; 11, 289; 12, BpPa1; 13, Bp7; 14, ATCC14707; 15, ladder with sizes (kb) in right margin.

elenans) and 285 and 289 (both from *P. menetriesi*), produced the same band pattern (266 and 289 are shown in Fig. 2). All of the remaining 11 Latin American isolates (292, 470, 479, 491, 492, 495, 499, 503, 510, 524, 525) differed from each other.

Among the 19 North American isolates, only strains BpPj2 and NRRL B-4145 gave identical band patterns. BpPj2 was isolated from *Popillia japonica* and B-4145 from an unidentified insect. The remaining 17 North American isolates (ATCC 14706, ATCC 14707, BpPa1, BpPj1, BpCb1, BpCh1, DNG2, DNG4, DNG11, DNG12, A8, NRRL B-2522, KLN1, KLN2, KLN3, Bp6, Bp7), and the 2 European isolates (H1 and NRRL B-4081) (both from *Melolontha melolontha*), and the New Guinean isolate (Bp1) all differed from each other. A few representatives of these are shown in Figs. 1 and 2.

The significance of these results is not that these particular isolates can for the most part be differentiated, but that the method would often be appropriate for identification of newly isolated strains and for their comparison to standard strains. For example, it may be possible to determine whether the strain registered by a pesticide manufacturer and upon which safety tests have been reported is actually the strain present in the commercial product. For a product seeded into soil, it may be possible to determine whether the bacteria recovered from infected insects in the future are the bacteria from the product or from an indigenous strain. For ecological and specificity studies, it should be possible to determine whether two different infected insect species in the same location are infected with the same or different bacterial strains. If infected with different strains, it would suggest specificity in infectivity. A number of tests based upon molecular biological methods (for example DNA hybridization and random amplified polymorphic DNA) are useful for species determination. Although PFGE does not appear to allow differentiation of the two species *P. popilliae* and *P. lentimorbus*, it may be useful for identification of individual isolates.

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