

## A PCR Detection Method for Rapid Identification of *Melissococcus pluton* in Honeybee Larvae

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Received 14 November 1997/Accepted 10 March 1998

*Melissococcus pluton* is the causative agent of European foulbrood, a disease of honeybee larvae. This bacterium is particularly difficult to isolate because of its stringent growth requirements and competition from other bacteria. PCR was used selectively to amplify specific rRNA gene sequences of *M. pluton* from pure culture, from crude cell lysates, and directly from infected bee larvae. The PCR primers were designed from *M. pluton* 16S rRNA sequence data. The PCR products were visualized by agarose gel electrophoresis and confirmed as originating from *M. pluton* by sequencing in both directions. Detection was highly specific, and the probes did not hybridize with DNA from other bacterial species tested. This method enabled the rapid and specific detection and identification of *M. pluton* from pure cultures and infected bee larvae.

European foulbrood (EFB) is a disease of honeybee larvae which kills them when they are 4 or 5 days old, mostly in the early summer when bee colonies are rapidly growing. There is often a well-defined seasonal outbreak followed immediately by a spontaneous recovery. When the larvae die, they often turn brown, decompose, and give off a foul odor which is associated with the disease. Several bacteria may be associated with cases of EFB, and most have at one time or another been considered as the primary pathogen (5). At various times the disease has been attributed to *Streptococcus apis* (12), *Bacillus alvei* (10), and *Bacterium eurydice* (8). Today it is accepted that EFB is caused by *Melissococcus pluton* (5), which is a gram-positive bacterium formerly known as *Bacillus pluton* (17, 18). The other bacteria previously thought to cause EFB are in fact commonly associated secondary bacteria which rapidly accelerate the death of the infected larvae.

The disease is widespread and has been of economic importance in North and South America, Europe, Japan, Australia, India, and southern Africa (13). The diagnosis of EFB is initially based on typical disease symptoms in the honeybee colony. If the disease is widespread, the comb takes on a pepper-box appearance, with many uncapped and diseased cells mixed with normal capped cells. Another symptom is that caps sealing healthy brood are convex while those of diseased brood are concave and sometimes punctured (16). Infected larvae first turn yellow and then brown and die twisted against the side of the cell or melted at the bottom of the cell. The remains of the larva dry out and form a scalelike protrusion in the cell. Worker, drone, and queen larvae are all susceptible to EFB.

Despite the widespread economic importance of EFB, the routine cultivation and identification of *M. pluton* remain problematic, as evidenced by the organism's being poorly described in *Bergey's Manual of Systematic Bacteriology* (10a). In addition, EFB is not routinely screened by honeybee disease laboratories worldwide, despite a number of publications comparing techniques for analysis of EFB (1, 11). Instead, these laboratories tend to use the presence of the secondary bacterial invaders *Achromabacter eurydice*, *Bacillus laterosporus*, and *B.*

*alvei* as presumptive evidence of the presence of *M. pluton* (11, 16). Although selective medium for the cultivation of *M. pluton* exists (2, 3, 5, 6, 7, 11), involving anaerobic incubation on yeast extract medium supplemented with sugar, starch, cysteine, and potassium, the cultural requirements are fastidious and *M. pluton* is extremely difficult to isolate. Competition from other bacteria is a problem, and even if isolated, *M. pluton* is difficult to identify due to its pleomorphic nature (16).

EFB has most commonly been diagnosed by the microscopic identification of *M. pluton* early in the infection cycle, before the appearance of secondary microflora associated with the disease. Negative staining with nigrosin assists in the identification of bacteria resembling *M. pluton* (1, 2). The bacterium is a non-spore-forming, lanceolate coccus, occurring singly, in pairs, and in chains and measuring 0.5 to 0.7 by 1.0  $\mu\text{m}$  (1, 16). *M. pluton* may also be distinguished by scanning electron microscopy (1), by enzyme-linked immunosorbent assays (Pinnock and Featherstone [14]), and with polyclonal antisera by using type strains (2).

No method for the identification of the causative organism of EFB has, however, become established in routine screening. In order to quickly and accurately identify the presence of *M. pluton*, PCR primers were designed from the 16S rRNA gene of *M. pluton*. The 16S rRNA genes are essential for the survival

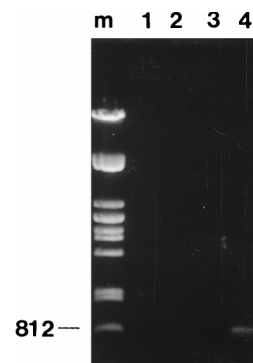


FIG. 1. Agarose gel (0.8%) showing PCR products from genomic DNA from different lysed bacterial species (pure cultures). Lanes: m, *Pst*-cut lambda DNA marker (the 812-bp band is indicated); 1, *E. coli*; 2, *B. alvei*; 3, *S. aureus*; and 4, *M. pluton*.

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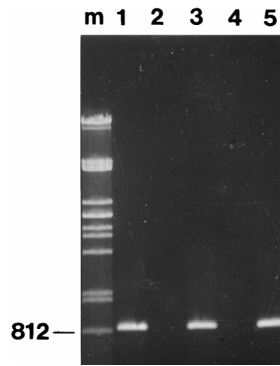


FIG. 2. Agarose gel (0.8%) showing PCR products from healthy and diseased bee larvae and an *M. pluton* pure culture. Lanes: m, *Pst*-cut lambda DNA marker (the 812-bp band is indicated); 1, *M. pluton* type strain; 2, nondiseased bee larvae; 3, diseased bee larvae; 4, nondiseased bee larvae; and 5, diseased bee larvae.

of all organisms and are highly conserved in all kingdoms; therefore, it is an ideal molecular marker to use for identification (20). However, to isolate the 16S rRNA gene by PCR and sequence it, in order to identify the organism from which it came, would be too time-consuming and expensive to perform on a routine basis. The PCR primers used here were designed from a region of the *M. pluton* 16S rRNA gene that was not homologous to other bacterial 16S rRNA gene sequences deposited in the available databases. The primers were designed to amplify a PCR amplicon which was 831 bp and would be unique to *M. pluton*.

The PCR primers were tested against four bacteria, including the *M. pluton* type strain (LMG 9003), *Escherichia coli*, *B. alvei*, and *Staphylococcus aureus* were grown in Luria broth, and *M. pluton* was grown in basal medium (4). Genomic DNAs were prepared according to standard methods (19). The DNA pellets were resuspended in 50 µl of 1× TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA). Approximately 1 to 3 µg of genomic DNA was amplified in a 50-µl reaction mixture in a Hybaid OMN-E thermocycler. The PCR was optimized with the following concentrations: 2 mM MgCl<sub>2</sub>, 50 pmol of primers (primer 1, 5' GAAGAGGAGTTAAAAGGCGC 3'; primer 2, 5' TTATCTCTAAGGCGTTCAAAGG 3') per µl, 25 mM

(each) deoxyribonucleoside triphosphate, and 1 U of *Taq* polymerase per µl. The PCR conditions consisted of a cycle of 95°C (1 min); 30 cycles of 93°C (1 min), 55°C (30 s), and 72°C (1 min); and a final cycle of 72°C (5 min). The molecular weights of the PCR products were determined by electrophoresis in a 0.8% agarose gel and staining with ethidium bromide. Under these PCR conditions only the *M. pluton* type strain produced a PCR product. As expected, this product banded alongside the 812-bp lambda *Pst* marker (Fig. 1).

The PCR primers were then used directly with honeybee larvae. Two bee larvae that showed symptoms of EFB disease and two apparently healthy worker larvae from Stellenbosch, South Africa, were tested. Each larva was incubated individually in the basal medium overnight, anaerobically, at 30°C in an anaerobic jar containing hydrogen plus 10% (vol/vol) CO<sub>2</sub>. Two milliliters of each sample was then centrifuged at 1,000 × g for 2 min, and the supernatant was centrifuged at 10,000 × g for 5 min. The resultant pellets were resuspended in 100 µl of sterile H<sub>2</sub>O and heated at 95°C for 15 min. One microliter was amplified in a 50-µl PCR mixture. When the resulting PCR products were run on agarose gels only the diseased larvae produced bands of approximately 812 bp, whereas the healthy larvae did not (Fig. 2).

To confirm that the bee larva PCR product was from *M. pluton*, the PCR product was cloned into pCR-Script Amp SK(+) cloning vector and transformed with the pCR-Script Amp SK(+) cloning kit (Stratagene). Plasmids were purified with the nucleobond kit (Maherey-Nagel) for plasmid isolation. The PCR product was sequenced in both directions by standard methods (15). A sequence similarity search was done by using the BLAST server at the National Center for Biotechnology Information. The PCR product was found to be 831 bp long, which was the same size as the region on the *M. pluton* 16S rRNA gene between the two primers. An alignment of the PCR product and the *M. pluton* 16S rRNA gene sequence showed the two sequences to have base pair mismatches at positions 593 and 824 of the 16S rRNA gene (Fig. 3).

The high degree of homology between the PCR product and the *M. pluton* 16S rDNA gene (99.7% [data not shown]) clearly indicates that the PCR product was from *M. pluton*. Previous studies have shown the 16S rDNA gene of *M. pluton* to exhibit the highest levels of sequence similarity with members of the *Enterococcus* genus (range, approximately 94 to 96%) (9). The



FIG. 3. Alignment of bases 84 to 913 of the 16S rRNA gene of *M. pluton* (NCBI accession no. X75752) with the PCR product obtained from enriched bee lysate. The primer-binding regions are indicated in boldface type, and the two mismatches at positions 593 and 824 are boxed.

PCR product used in this study was found to have its closest sequence similarity to the *Enterococcus faecium* 16S rRNA gene (96.3% [data not shown]). More significantly, the PCR primers used here, compared to *E. faecium*, had six and five mismatches over the 20-mer regions of primer 1 and primer 2, respectively. Since *Enterococcus* is the genus most closely related to *Melissococcus*, it is not possible that these bacteria were detected with these PCR primers.

The technique described here is a quick and accurate method for the identification of *M. pluton* directly from diseased bee larvae. The time required to complete the procedure, following overnight incubation of the bee larvae, is only 6 h, thus making the method an ideal diagnostic technique. This is a particularly useful technique because of the internationally recognized difficulty in isolating and identifying *M. pluton*.

This work was partly funded by the Foundation for Research Development.

We are grateful for sequencing carried out by Di James from the Department of Microbiology at the University of Cape Town.

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