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Development of a group-specific PCR combined with ARDRA for the identification of *Bacillus* species of environmental significance

Xi-Yang Wu[a,b], Mark J. Walker[b], Michael Hornitzky[a], James Chin[a,*]

[a]*Immunology and Microbiology, Elizabeth Macarthur Agriculture Institute, NSW DPI, PMB 8, Camden, NSW 2570, Australia*
[b]*School of Biological Sciences, University of Wollongong, Wollongong, NSW 2522, Australia*

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Abstract

A group-specific primer pair was designed to amplify the 16S rRNA gene of representative reference strains from environmentally sourced, mesophilic aerobic spore-forming *Bacillus* taxa. The PCR generated a 1114 bp amplicon but did not do so with DNA extracted from 16 other Eubacterial species. When amplicons were digested with restriction enzymes *Alu*I or *Taq*I, different profiles containing between 2 and 5 fragments ranging in size from 76 to 804 base pairs were seen with different *Bacillus* species. This procedure, known otherwise as amplified ribosomal DNA restriction analysis or ARDRA, produced unique and distinguishable patterns to differentiate between 15 ATCC reference strains (10 *Bacillus*, 3 *Paenibacillus* and 2 *Brevibacillus* member species) as well as 3 misidentified *Bacillus* probiotic strains in a commercial collection. Our simplified PCR-ARDRA protocol provides a facile method for the identification of most environmentally important species of *Bacillus*.

Keywords: *Bacillus*-specific primers; ARDRA; 16S rDNA gene; *Bacillus* identification

1. Introduction

Members of the genus *Bacillus* have played a significant dual role in many human activities. On the one hand, species such as *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* are used industrially for the production of enzymes, antibiotics, solvents and other molecules (Gerhartz, 1990). Others such as *B. thuringiensis* and *B. sphaericus*, because of their insecticidal activity, are used in crop protection (Bourque et al., 1995) while *B. mycoides* has the ability to promote plant growth (Petersen et al., 1995). In countries like Italy and Vietnam, *B. subtilis*, *B. clausii* or *B. alcalophilus* have been used as an oral bacteriotherapeutic for the treatment of gastrointestinal disorders (Casula and Cutting, 2002; Hoa et al., 2000; Senesi et al., 2001). On the other hand, some strains of *B. anthracis* and *B. cereus* are pathogenic to humans and/or animals (Shangkuan et al., 2000) and honeybees are susceptible to American foulbrood, a disease

* Corresponding author. Tel.: +61 2 46406359; fax: +61 2 46406384.
E-mail address: james.chin@dpi.nsw.gov.au (J. Chin).

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caused by *Paenibacillus larvae* subsp. *larvae* (formerly *Bacillus larvae*) (White, 1920).

In its original classification, the genus *Bacillus* contained a heterogeneous assembly of aerobic, or facultatively anaerobic, Gram-positive, rod-shaped, spore-forming bacteria widely distributed in the environment. Traditionally, *Bacillus* spp. are identified in the laboratory by biochemical tests and fatty acid methyl ester (FAME) profiling (Bobbie and White, 1980; Vaerewijck et al., 2001). Alternatively, the API (Analytab Products, Inc.) system of identification has been shown to be more reproducible than classical methods (Logan and Berkeley, 1984) and is capable of speciating bacilli using a combination of the 12 tests in the API 20E strip, and 49 tests in the API 50CHB strips. These phenotyping protocols are laborious and time-consuming to undertake and cannot provide a rapid screening system (Wattiau et al., 2001). The shortcomings of phenotypically-based identification methods have led to the development of molecular alternatives based on the microbial genotype or DNA sequence. This approach minimizes problems associated with typability and reproducibility, and importantly, facilitates the assembly of large reference databases (Olive and Bean, 1999).

Comparisons of the 16S rRNA sequence is one of the most powerful tools for the classification of microorganisms (Wang et al., 2003; Woese, 1987; Yamada et al., 1997) and have provided sequence specific primers as gold standards for the identification of pure cultures of *Bacillus* species such as *B. subtilis* (Wattiau et al., 2001), *B. cereus* and *B. thuringiensis* (Hansen et al., 2001), and *Paenibacillus alvei* (formerly *Bacillus alvei*) (Djordjevic et al., 2000). However, environmental samples such as those from sewage, water, soil, feces and even beehives, usually contain mixtures of *Bacillus* species. The presence of such combinations can be better detected with the use of a group-specific primer that distinguishes as many member species as possible within the genus. Individual species representative of different *Bacillus* genera can then be characterised by subjecting the amplicons to restriction enzyme digest. Although genus-specific primers have been successfully developed for lactobacilli (Dubernet et al., 2002), mycoplasmas (van Kuppeveld et al., 1992), *Bifidobacterium* (Matsuki et al., 1999), *Pandoraea* (Coenye et al., 2001) and *Clostridium* (Van Dyke and McCarthy, 2002), a group-specific primer pair capable of amplifying a specific sequence of 16S rDNA from all *Bacillus* taxa has not been developed in accompaniment with restriction digest mapping.

In this study, we have focused on a subset of environmental (probiotic and gut-associated) *Bacillus* strains that are primarily mesophilic spore-forming bacteria capable of growing aerobically on nutrient agar at neutral pH, and between 25–45 °C. The strains of interest to us, included species from related genera such as *Bacillus*, *Brevibacillus*, *Paenibacillus* and *Geobacillus* but not *Alicyclobacillus* (thermophiles and acidophiles), *Amphibacillus* and *Ureibacillus* (alkaliphiles), *Halobacillus*, *Filobacillus*, *Gracilibacillus*, *Virgibacillus* and *Salibacillus* (halotolerant or halophilic bacteria) and even several extremophilic, asporogenous and nutritionally fastidious species present in *Bacillus*, *Geobacillus* and *Paenibacillus*. The specificity of our target PCR primers was also validated against related Gram-positive but non-*Bacillus* species that frequently co-exist with *Bacillus* in the environment. Species identification of *Bacillus*, based on amplicons generated by our group-specific PCR primer pair, was accomplished by amplified ribosomal DNA restriction analysis (ARDRA) with *Alu*I and *Taq*I. The utility of our assay was then used to check for mislabelled identifications if any, in a commercial collection of probiotic *Bacillus* strains.

2. Materials and methods

2.1. Reference strains and culture conditions

Reference strains used in this study are listed in Table 1 including 15 reference *Bacillus* strains; 18 reference non-*Bacillus* strains; 17 reference probiotic strains; 50 reference honeybee strains. Reference strains are defined as those strains that have been speciated using standard taxonomical protocols by ATCC, commercial probiotic companies and diagnostic laboratories.

Bacillus strains were cultivated in tryptic soy broth (TSB) or agar (TSA, DIFCO, Becton Dickinson, Sparks, MD) aerobically at 34 °C. The probiotic *Bacillus* strains were recovered by suspension of dried bacterial preparations in saline followed by plating on TSA. The non-*Bacillus* strains were cultivated on