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Evidence for plasmid-mediated tetracycline resistance in *Paenibacillus larvae*, the causal agent of American Foulbrood (AFB) disease in honeybees

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Abstract

Paenibacillus larvae is the causal agent of American Foulbrood (AFB) disease, the most virulent bacterial disease of honeybee (*Apis mellifera* L.) brood. Oxytetracycline is the main antibiotic used for prevention and control of AFB. Using the polymerase chain reaction, isolates were screened for the presence of the tetracycline resistance *tet*(K) and *tet*(L) determinants. Four isolates (5%), which correlated with the Tc-resistant phenotypes, were found to carry the *tet*(K) determinant, whereas none carried the *tet*(L) determinant. *P. larvae* cells were also screened for the presence of extrachromosomal DNA and evidence obtained that tetracycline resistance is plasmid-encoded. A few *P. larvae* isolates were found to be able to transfer the *tet*(K) determinant to *Bacillus subtilis*, suggesting that a conjugation mechanism may be involved in the transfer of the tetracycline-resistant phenotype. Minimum inhibitory concentrations to tetracycline were determined for 75 isolates of *P. larvae* from different geographical origins and found to range between 0.062 and 128 μg tetracycline ml^{-1}, with MIC_{50} and MIC_{90} values of 1 and 4, respectively. According to results from *P. larvae* populations, isolates could be considered as susceptible when their MICs were <4, intermediate for MICs values 4–8 and resistant for MICs ≥16. To our knowledge, this is the first report of Tc^r *Paenibacillus* species carrying a *tet*(K) gene, and also the first record of *P. larvae* strains carrying *tet*(K) determinants and its correlation with the presence of extrachromosomal DNA.

Keywords: AFB; *Paenibacillus larvae*; Tetracycline resistance; Plasmids; Honeybees

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1. Introduction

American Foulbrood disease (AFB) caused by the spore-forming bacterium *Paenibacillus larvae* (Genersch et al., 2006) is a highly contagious disease affecting the larval and pupal stages of honeybees (*Apis mellifera* L.). AFB is the most widespread and destructive of the brood diseases, and possesses unique problems for prevention and control because the spores can remain viable for long periods of time and survive adverse environmental conditions (Matheson and Reid, 1992; Shimanuki and Knox, 2000). It was first diagnosed in Argentina in 1989 and, since then, it has become widespread in all the beekeeping areas, particularly in Buenos Aires Province where the incidence is higher than 50% (Alippi et al., 2004).

In many countries, e.g. UK and New Zealand, AFB-affected colonies are always destroyed by burning the bees, brood combs and all movable parts; the hive box and other components, such as queen excluders, are often sterilized by scorching, gamma irradiation or immersion in hot paraffin (Matheson and Reid, 1992; Goodwin and Van Eaton, 1999). In other countries, such as Argentina, USA and Canada, colonies are treated with antibiotics that suppress clinical symptoms by controlling proliferation of only vegetative cells, but bacterial spores accumulate within the hive and contaminate honey, remaining infective for many years. This may lead to recurrence of AFB later in the life of the colony (Oldroyd et al., 1989). In areas were disease incidence is high, antibiotic treatment is a possible alternative to the burning of infected beehives. Oxytetracycline and tylosin are the only antibiotics currently approved for its use in the prevention and control of AFB in honeybee colonies; however, oxytetracycline-resistant *P. larvae* isolates have been detected in certain areas of the USA, Canada and Argentina (Evans, 2003; Miyagi et al., 2000).

Resistance to tetracyclines (oxytetracycline, chlortetracycline, metacycline, doxycycline, minocycline) is frequently due to the acquisition of new genes, which are associated with either mobile plasmids or transposons (Roberts, 1996, 2005). There are three main mechanisms underlying tetracycline resistance: energy-dependent efflux of tetracycline by a proton antiporter system, ribosomal protection, and enzymatic inactivation of the tetracycline molecule (Roberts, 1996). The two first mechanisms are widely distributed among Gram negative and also on Gram positive bacteria. Up to now, 38 different *tet* and *otr* genes have been reported for Gram positive bacteria, whereas only the occurrence of determinants *tet*(K), *tet*(L), *tet*(M) and *tet*(W) have been reported in *Bacillus* and its related species (Chopra and Roberts, 2001; Roberts, 2005; Villedieu et al., 2003).

Even though the occurrence of oxytetracycline (OTC)-resistant strains of *P. larvae* has been reported (Evans, 2003; Miyagi et al., 2000), there are no studies on the tetracycline resistance determinants of *P. larvae*-resistant strains. It has been reported that OTC resistance in *P. larvae* does not correlate with bacterial haplotype, suggesting that either resistance has evolved numerous times during microbial evolution or that resistance involves recent events in horizontal transference via a non-genomic route (Evans, 2003).

The aims of the present work were to investigate the molecular basis of tetracycline resistance determinants of *P. larvae* and to assess the susceptibility of 75 strains of *P. larvae* from diverse geographical origins to tetracycline via minimal inhibitory concentration (MIC) and disk diffusion method. As there are no standards in the NCCLS or CLSI guidelines (CLSI, 2005; NCCLS, 2002, 2003), a second objective was to define cut-off points between susceptible and resistant strains of the Foulbrood pathogen as well as interpretative criteria.

2. Materials and methods

2.1. Bacterial strains

A total of 75 *P. larvae* isolates from diverse origins, used in this study, are listed in Table 1; the collection includes six *P. larvae* strains from culture collections. *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213, from the American Type Culture Collection (ATCC), were used for quality control in susceptibility testing. Additional strains of *P. larvae* and *Bacillus subtilis,* used for mating experiments, and transconjugants are listed in Table 2.

All isolates were stored frozen at −80 °C in liquid MYPGP (Dingman and Stahly, 1983) plus 20% glycerol (v/v).