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Genetic analysis of housekeeping genes of members of the genus *Acholeplasma*: Phylogeny and complementary molecular markers to the 16S rRNA gene

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Abstract

The partial nucleotide sequences of the *rpoB* and *gyrB* genes as well as the complete sequence of the 16S–23S rRNA intergenic transcribed spacer (ITS) were determined for all known *Acholeplasma* species. The same genes of *Mesoplasma* and *Entomoplasma* species were also sequenced and used to infer phylogenetic relationships among the species within the orders *Entomoplasmatales* and *Acholeplasmatales*. The comparison of the ITS, *rpoB*, and *gyrB* phylogenetic trees with the 16S rRNA phylogenetic tree revealed a similar branch topology suggesting that the ITS, *rpoB*, and *gyrB* could be useful complementary phylogenetic markers for investigation of evolutionary relationships among *Acholeplasma* species. Thus, the multilocus phylogenetic analysis of *Acholeplasma multilocale* sequence data (ATCC 49900 (T) = PN525 (NCTC 11723)) strongly indicated that this organism is most closely related to the genera *Mesoplasma* and *Entomoplasma* (family *Entomoplasmataceae*) and form the branch with *Mesoplasma seiffertii*, *Mesoplasma syrphidae*, and *Mesoplasma photuris*. The closest genetic relatedness of this species to the order *Entomoplasmatales* was additionally supported by the finding that *A. multilocale* uses UGA as the tryptophan codon in its *gyrB* and *gyrA* sequences. Use of the UGA codon for encoding tryptophan was previously reported as a unique genetic feature of *Entomoplasmatales* and *Mycoplasmatales* but not of *Acholeplasmatales*. These data, as well as previously published data on metabolic features of *A. multilocale*, leads to the proposal to reclassify *A. multilocale* as a member of the family *Entomoplasmataceae*.

Keywords: Mollicutes; *rpoB* and *gyrB* genes; Mycoplasmas

1. Introduction

The bacterial genus *Acholeplasma* of the order *Acholeplasmatales*, family *Acholeplasmataceae*, currently includes 15 species: *A. axanthum*, *A. brassicae*, *A. cavigenitalium*, *A. equifetale*, *A. granularum*, *A. hippikon*, *A. laidlawii*, *A. modicum*, *A. morum*, *A. multilocale*, *A. oculi*, *A. palmae*, *A. parvum*, *A. pleciae*, and *A. vituli* (Woese et al., 1980; Neimark and London, 1982; Rogers et al., 1985; Pollack et al., 1996a; Angulo et al., 2000; Knight, 2004) (http://www.ncbi.nlm.nih.gov/Taxonomy/). *Acholeplasma* species are widely distributed in the nature and can be detected and isolated from different plant, avian, and mammalian sources (Tully, 1984; Razin et al., 1998; Ayling et al., 2004). The *Acholeplasma* members are chemoorganotrophs and depend on carbohydrates and amino acids but, in contrast to the species of the family *Mycoplasmataceae* and *Spiroplasmataceae* do not require sterol for growth. The range of the growth temperature varies from 25 to 37 °C (Rogers et al., 1985; Razin et al., 1998). All *Acholeplasma* species except for *A. multilocale* reduce the redox indicator

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benzyl viologen (Pollack et al., 1996a). The genome sizes of *Acholeplasma* range from 1215 kb (*A. brassicae*) to 2095 kb (*A. vituli*) with the GC contents vary from 28.3% (*A. oculi*) to 38.3% (*A. vituli*) (Tully et al., 1994; Artiushin et al., 1995; Angulo et al., 2000). *Acholeplasma* taxonomy is currently based on phenotypic criteria, predominantly biochemical and serological features, as well as genetic methods that include DNA–DNA hybridization and analysis of the 16S rRNA gene sequence relationships (Stephens et al., 1983; Razin et al., 1998; Knight, 2004). The application of the serological methods alone for *Acholeplasma* species identification may be complicated due to the previously reported interspecies cross-reactivity of antibodies (Tully, 1984). The phenotypic, predominantly metabolic, inter and intragenus *Acholeplasma* differentiation is based on the use of the following biochemical features: non-requirement of sterol for growth, fermentation of mannose, esculin and arbutin hydrolysis, carotenoid pigment production, and medium acidification due to the catabolism of some sugars (Tully, 1984; Pollack et al., 1996b; Pollack et al., 1997). Overall, in comparison with the currently available DNA identification methods, the traditional serological and biochemical identification of *Acholeplasma* species is laborious, time-consuming and restricted to specialized laboratories. As a result, the development of novel and simplified approaches capable of precisely identifying a bacterial species is highly desirable. Thus, investigations of intra and interspecies divergences of multiple chromosomal loci of *Mollicutes* provide valuable information for comprehensive genetic characterization of these organisms, species definition, and taxa classification (Stackebrandt et al., 2002; Drancourt and Raoult, 2005; Maiden, 2006).

The recognized divergence among the *Mollicutes* has substantially increased over the past decade through studies of the 16S rRNA gene sequences and the use of differences in this gene for taxonomic placement of *Mollicutes* isolates, particularly species which represent fastidious or uncultivated forms (Neimark et al., 2001; Messick et al., 2002; Neimark et al., 2002; Knight, 2004). It is noteworthy that a study of the 16S rRNA gene had a significant impact on the understanding of *Mollicutes* evolution, taxonomy, and ecological distribution as well (Woese et al., 1980; Razin et al., 1998; Messick et al., 2002; Gasparich et al., 2004). Recently, on the basis of the 16S rRNA phylogenetic analysis, *Mesoplasma* and *Entomoplasma* species were proposed to be combined into a single genus, presumably with the name of *Entomoplasma* (Johansson, 2000; Gasparich et al., 2004). For this study, we will refer to these species as the *Mesoplasma*/*Entomoplasma* phylogenetic group of organisms and will use the official binomial species names when referring to specific organisms.

The 16S rRNA gene is a commonly accepted genetic marker for taxonomic identification of bacteria. However, in some cases the use of it as a marker might be complicated due to the presence of multiple ribosomal RNA (*rrn*) operon copies in a single bacterial genome (Acinas et al., 2004) or the low interspecies polymorphism in some taxonomic groups (Boyer et al., 2001; Clarridge, 2004; Schloss and Handelsman, 2004), including *Mollicutes* (Gundersen et al., 1994; Pettersson et al., 2000; Kim et al., 2003; Chalker and Brownlie, 2004; Gasparich et al., 2004). Previously published data showed that *Acholeplasma* species have two *rrn* operons (Neimark, 1983; Amikam et al., 1984; Razin et al., 1984; Razin et al., 1998) that differ from each other in length and sequence of their 16S–23S rRNA intergenic transcribed spacer (ITS) regions (Nakagawa et al., 1992).

Recently, the genetic relatedness between different bacterial taxons was successfully tested by multilocus sequence typing based on simultaneous analysis of several housekeeping genes (Holmes et al., 2004; Rubin et al., 2005). The potential candidates for such markers are the *rpoB*, *gyrB*, elongation factor Tu (*EF-Tu*), phosphoglycerate kinase (*pgk*), and heat shock protein (*dnaK*) genes, as well as the 16S–23S rRNA intergenic transcribed spacer region (Kamla et al., 1996; Johansson, 2000; Kim et al., 2003; Wolf et al., 2004; Drancourt and Raoult, 2005).

The main goal of this study was to assess the advantage of using the ITS, *rpoB*, and *gyrB* genes for reconstructing the phylogeny of *Acholeplasma* species, as well as *Mesoplasma* and *Entomoplasma* species, which share some genetic features with members of the genus *Acholeplasma*.

2. Materials and methods

2.1. Mollicutes strains and culture

The *Acholeplasma* strains (Table 1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and the *Mollicutes* Collection at Purdue University (Purdue University, West Lafayette, IN). The broth and agar for *Mollicutes* growth were as described in previous studies (Tully, 1984; Volokhov et al., 2006).

2.2. Genomic DNA isolation

Genomic DNA was isolated either from *Mollicutes* culture or directly from a small part of lyophilized samples using the DNeasy Tissue Kit (Qiagen, Chatsworth, Calif.), according to the manufacturer's protocol. DNA concentrations in solutions were calculated on the basis of their optical density at 260 nm.

2.3. PCR amplification of 16S–23S rRNA ITS region

Broad-spectrum *Mollicutes*-specific primers for PCR amplification of 16S–23S rRNA ITS region were designed using sequences in the 16S and 23S rRNA genes conserved for most *Firmicutes* (Volokhov et al., 2006). Forward PCR primer 16S-F-MYC (GGTGAATACGTTCTCGGGTC TTGTACACAC) and reverse primer 23S-R1-MYC (TNCTTTTCACCTTTCCCTCACGGTAC) were used for amplification of the ITS from all the *Mollicutes* species used in the study (Table 1). The standard PCR mixture