



Reclassification of the pathogen for empty-gut disease of Chinese oak silkworm, *Antheraea pernyi*

Lin-Ling Wang¹, Feng Qin¹, Ce Song² and Ze-Yang Zhou^{1*}

¹ College of Life Sciences, Chongqing Normal University, Chongqing, China, 400047. ² Liaoning Silkworm Scientific Research Institute, Dandong, Liaoning, China, 118100. *e-mail: zyzhou@cqnu.edu.cn

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Abstract

Empty-gut disease is a common disease of Chinese oak silkworm, *Antheraea pernyi* (Tussah). The study on the pathogen of the disease is of great help in prevention and cure of the disease. Previous studies showed that the pathogen was a new species in the genus *Streptococcus*, which was therefore named as *Streptococcus pernyi* sp.nov. In this study, with the purified pathogen, we performed some physiological and biochemical experiments, and also cloned and sequenced 16S rRNA gene of the pathogen. Subsequently, we analyzed its similarity and genetic distance with other related bacteria by using DNASTAR software, and a NJ tree was then constructed for phylogenetic analysis by MEGA 3.0. Unexpectedly, the results showed that the pathogen causing empty-gut disease of Tussah belongs to the genus *Enterococcus* with 99% bootstrap support. Therefore, *Streptococcus pernyi* sp.nov should be renamed as *Enterococcus pernyi*. Reclassification of the pathogen should keep pace with the development of modern taxonomy. In doing so, it will better our understanding of the pathogen and contribute to further study of the pathogen.

Key words: Empty-gut disease of Tussah, pathogen, 16S rRNA gene, *Streptococcus pernyi* sp.nov, *Enterococcus pernyi*.

Introduction

Chinese oak silkworm, *Antheraea pernyi* (Tussah) is a silk-spinning insect whose larvae live mainly on leaves of oak (*Quercus*), *Juglans regia* Linn., *Cinnamomum camphora* (L.) Presl., *Fructus Crataegi* Pinnatifidae, etc. China is the first country to rear and make use of Tussah, with rearing areas currently distributed in more than 10 provinces and regions. Tussah silk is not only the raw material for Tussah cloth but also very useful in industries and national defense.

With its high incidence and large affected area, empty-gut disease seriously affects the production of Tussah. In the 1950s and 1960s, the annual average incidence of the disease in China was between 30% and 40%, with the most serious year reaching up to 70%. In recent years, with researches into and popularization of methods for prevention and cure of the disease, the incidence has dropped to some extent. However, the incidence is still high. For example, it reached up to between 25% and 40% and showed a rising tendency in Liaoning province^{5,13}.

In 1979, Yu¹⁶ characterized the pathogen causing the disease and found that while the pathogen was consistent with the genus *Streptococcus* in terms of morphology, physiology, biochemistry, etc, it was different in serological reaction from identified species belonging to the genus. Therefore, the pathogen was established as a new species in the genus *Streptococcus* and named as *Streptococcus pernyi* sp.nov^{15,16}.

In this study, we performed some physiological and biochemical experiments and also cloned and sequenced 16S rRNA gene of the pathogen. After phylogenetic analysis of the 16S RNA gene, we concluded that *Streptococcus pernyi* sp.nov should be renamed as *Enterococcus pernyi*.

Materials and Methods

Materials: A pathogenic strain was obtained from Sericultural Research Institute of Liaoning, and another pathogenic strain was isolated from a diseased Tussah by College of Life Sciences, Chongqing Normal University.

Morphological observation and physiological and biochemical characterization: Experiments were performed according to methods described in Bergey's Manual of Determinative Bacteriology.

Extraction of genomic DNA of the pathogen and amplification and sequencing of its 16S rRNA gene: SDS 10% was added to the pathogen to a final concentration of 0.5% and then the tube was placed in ice for 15 min. Subsequently, 20 mg/ml proteinase K was added to a final concentration of 200 µg/ml, and then the tube was incubated in water bath at 55°C for 4.5 h. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed well, and then the mixture was centrifuged at 10,000 rpm for 5 min. Aqueous phase was transferred to a new tube and mixed well with 0.1 volume of 3 M NaOAc and 2.5 volumes of 100% cold ethanol, and then the tube was placed at -20°C for 30 min, followed by centrifugation at 12,000 rpm for 20 min. Supernatant was discarded and pellet was rinsed once with 1 ml of 75% cold ethanol and then air dried. TE 50 µl was used to dissolve the extracted DNA, and the resulting DNA solution was stored at -20°C for later use. According to conserved sequences of ribosome 16S rRNA gene, primers were designed as following: forward primer (GAGTTGCGAACGGGTGAG); reverse primer (CTTGTTACGACTTCACCC). PCR was performed under the

following conditions: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 40 s at 55 °C and 1 min at 72 °C, and a final extension at 72 °C for 10 min. PCR product was ligated with PMD18-T and transformed into DH5 α , and then plasmid was extracted and sequenced by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd and Shanghai Boshang biotechnology Co., Ltd.

Phylogenic analysis: To obtain similarity and genetic distance, multiple sequence alignment was carried out by using Jotun hein method of DNASTar software. ClustalX version 1.8¹⁴ was used to perform multiple sequence alignment of 16S rRNA sequences. MEGA 3.0⁸ was used to construct NJ tree, with Kimura 2-parameter being the distance parameter and bootstrap value being 1000 bootstrap replicates.

Results

Morphology, physiology and biochemistry of the pathogen: The pathogen, negative in catalase test, was a Gram-positive facultative coccus, arranged in pair or short chain without spores in liquid medium. Moreover, it could be grown in liquid medium containing 6.5% NaCl. In rabbit blood medium, hemolysis was induced by the pathogen, whereas no hemolysis was induced in sheep blood medium.

16S rRNA gene and phylogenic analysis of the pathogen: With the genomic DNA of the pathogen as template, its 16s rRNA gene was PCR amplified. After being sequenced by 5 times, the 16S rRNA gene of the pathogen, whose size is 1422 bp and GC content is 53.31%, has been submitted to GenBank database of NCBI (<http://www.ncbi.nlm.nih.gov/Genbank/>) under the accession number FJ555518.

Multiple sequence alignment was carried out by using Jotun hein method of DNASTar software, and similarity and genetic distances were obtained (see Table 1). From the table, we can see that 16S rRNA gene of the pathogen shows 99.6% similarity with that of the *Enterococcus* isolated from *Bombyx mori* (silkworm) by Zhejiang university, 99.4% with that of the *Enterococcus mundtii* and 96.3% with that of *Enterococcus faecalis*, the type species of *Enterococcus*. On the contrary, 16S rRNA gene of the pathogen shows only 89.3% similarity with that of *Streptococcus pyogenes*, the type species of *Streptococcus*, 87.3% with that of *Streptococcus pneumoniae*, and 88.1% with that of *Streptococcus suis*. Therefore, 16S rRNA gene of the pathogen shows higher similarity and smaller genetic distance with those of the genus *Enterococcus*.

To further analyze the evolutionary relationship between the pathogen and other species, more 16S rRNA genes were selected from species in the genera *Enterococcus* and *Streptococcus*. After multiple sequence alignment through ClustalX, MEGA software

was used to construct NJ tree (Fig.1). From the NJ tree, we can see that the two genera are divided well into two branches and that the pathogen belongs to the genus *Enterococcus* with high bootstrap support. In the *Enterococcus* branch, the pathogen and *Enterococcus mundtii* are divided into two sub-branches with 98% bootstrap support, suggesting that they belong to different species. The pathogen and the *Enterococcus* isolated from silkworm by Zhejiang University belong to different species with 81% bootstrap support.

As shown above, the pathogen belongs to the genus *Enterococcus* with 99% bootstrap support. Therefore, *Streptococcus pernyi* sp.nov should be renamed as *Enterococcus pernyi*.

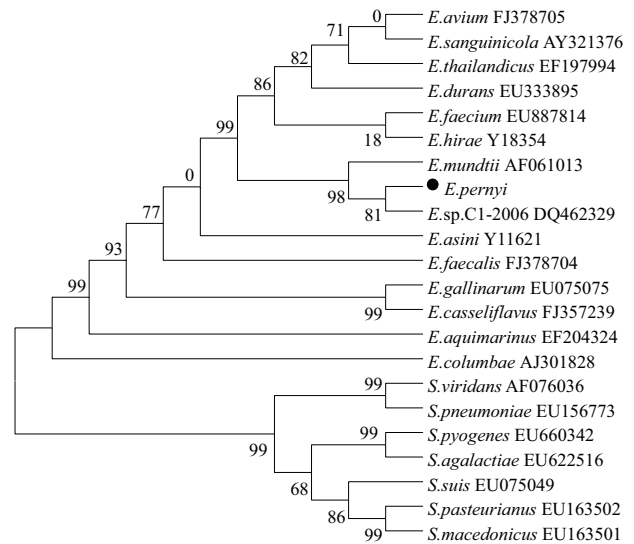


Figure 1. The NJ tree of 16S rRNA genes. Bootstrap percentages with 1000 resamplings appear above the branches; E. stands for *Enterococcus*, and S. for *Streptococcus*. GenBank Accession number for each sequence is shown next to the corresponding species name.

Discussion

16S rRNA gene is a relatively conserved gene existing in almost all prokaryotic organisms, and can be used as molecular clock due to its constant mutation rate in evolution. There are relatively variable regions in 16S rRNA gene, which provide important information for evolutionary relationship between species. Therefore, 16S rRNA sequence can be used as the tag for identifying species and the basis for classification in modern taxonomy.

In taxonomy, *Enterococcus* was originally classified as a Gram-positive coccus in the intestine and then into the genus

Table 1. Similarity and genetic distances among 16S rRNA genes of the pathogen and other species.

Divergence	Percent identity							
	<i>E. pernyi</i>	<i>E.sp. C1-2006</i>	<i>E. mundtii</i>	<i>E. faecium</i>	<i>E. faecalis</i>	<i>S. pneumoniae</i>	<i>S. pyogenes</i>	<i>S. suis</i>
<i>E. pernyi</i>		99.6	99.4	98.8	96.3	87.3	89.3	88.1
<i>E. sp. C1-2006</i>	0.4		99.9	99.1	95.6	87.9	88.6	88.0
<i>E. mundtii</i>	0.6	0.1		99.2	95.9	87.5	89.1	88.0
<i>E. faecium</i>	1.2	0.9	0.8		96.3	87.8	89.2	87.9
<i>E. faecalis</i>	3.8	4.5	4.3	3.8		87.4	88.8	86.6
<i>S. pneumoniae</i>	13.9	13.2	13.8	13.4	13.8		92.3	94.2
<i>S. pyogenes</i>	11.6	12.4	11.8	11.7	12.1	8.2		93.3
<i>S. suis</i>	13.1	13.2	13.2	13.3	14.8	6.1	7.1	

Streptococcus. With the establishment of Laneefield serological grouping method in 1930, Enterococcus was classified as group D *Streptococcus*. However, there was non-enterococcus group D *Streptococcus*. To accurately differentiate between Enterococcus and Streptococcus, Sherman proposed the use of the name, genus *Enterococcus*¹². In 1984, based on DNA hybridization experiment and sequence analysis of 16S rRNA genes, Shleifer and Kilpper-Balz separated Enterococcus from the genus *Streptococcus* and established the genus *Enterococcus* in which there were then only 2 species¹¹, *Enterococcus faecalis* and *Enterococcus faecium*. Subsequently, a number of new members of the genus *Enterococcus* were separated and identified^{1,3,4,6,7,9,10}. Currently, at least 42 species belonging to the genus *Enterococcus* are listed in NCBI Taxonomy (<http://www.ncbi.nlm.nih.gov/Taxonomy>).

In 1979, Yu *et al.*¹⁶ classified the pathogen into the genus *Streptococcus* and named it as *Streptococcus pernyi* sp.nov. Considering the limitation of classification criteria that prevailed at that time, their classification was correct. However, with the development of taxonomy, many bacteria have been reclassified. For example, *Streptococcus faecalis*, *Streptococcus faecium* and *Streptococcus avium* were renamed as *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus avium*, respectively^{6,11}. In China, the pathogen is still thought as an Streptococcus, and therefore, it is studied in the same manner as Streptococcus, seriously affecting the development of research into and prevention and cure of empty-gut disease of Tussah. Reclassification of the pathogen will better our understanding of the pathogen and contribute to further study of the pathogen.

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