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Original Research

Differentiation Among *Rodentibacter* Species Based on 16S–23S rRNA Internal Transcribed Spacer Analysis

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The internal transcribed spacer (ITS) regions of *Rodentibacter pneumotropicus*, *R. heylii*, *R. rarus*, *R. ratti*, and *R. heidelbergensis* and of a *Rodentibacter*-related β-hemolytic *Pasteurellaceae* taxon isolated from laboratory rodents were studied for their feasibility to discriminate among these species. The 6 species analyzed showed species-specific ITS patterns that were shared by the type strains and clinical isolates and that allowed their identification. Nevertheless, differentiating between the ITS band patterns of *R. pneumotropicus* and *R. ratti* is visually challenging. In all species tested, sequence analysis of the ITS fragments revealed a larger ITS$^{ile+ala}$, which contained the genes for tRNA$^{Ile(GAU)}$ and tRNA$^{Ala(UGC)}$, and a smaller ITS^{glu} with the tRNA$^{Glu(UUC)}$ gene. The ITS sequences varied among the 6 species evaluated, displaying identity levels ranging from 62% to 86% for ITS$^{ile+ala}$ and 68% to 90% for ITS^{glu}. Overall, ITS amplification proved to be a reliable method to differentiate among these important *Pasteurellaceae* species of laboratory rodents. Moreover, the ITS sequence variations recorded here might facilitate the design of probes for specific identification of these species. The ability to diagnose these organisms to the species level could increase our understanding of their clinical significance.

Abbreviations: ITS, internal transcribed spacer

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The members of the *Pasteurellaceae* isolated from rodents are among the most prevalent bacterial agents from experimental animal facilities.[18] Recently, [*Pasteurella*] *pneumotropica* and its closely related rodent *Pasteurellaceae* were reclassified into 8 distinct species and 2 genomospecies within the new genus *Rodentibacter*.[1] The uncertain taxonomic position of [*P.*] *pneumotropica* complex has hindered understanding of the epidemiology, pathogenesis, diagnosis, and control of infections caused by these microorganisms.[8] Currently *R. pneumotropicus* and *R. heylii* are considered to have tropism mainly toward mice, whereas *R. ratti* and *R. heidelbergensis* are rather rat-specific.[8,17] Despite their relatedness, isolates of *Rodentibacter* spp. of rat origin infected only a few mice by contact and experimentally, whereas mouse isolates infected all rats, thus showing that the rat isolates are more species specific than the mice isolates.[15] We recently documented that *R. heylii* naturally infects both rats and mice.[7] In addition to the known *Rodentibacter* species, a *Rodentibacter*-related β-hemolytic taxon is apparently often present in laboratory mice.[6,12] The members of the former [*P.*] *pneumotropica* complex (now *Rodentibacter* spp.) are generally regarded as classic opportunistic pathogens of rodents. However, little is known about the pathogenicity of the individual bacterial species.[8]

The new taxonomic separation of the previous *[P.] pneumotropica* complex into *Rodentibacter* species supports better diagnostic assays among this complex group of bacteria. Nevertheless, simple molecular techniques to differentiate these organisms are currently available only for *R. pneumotropicus* and *R. heylii*; 16S rRNA gene sequencing is the only alternative for the remaining *Rodentibacter* species.

The internal transcribed spacer (ITS) region has been used as a target for PCR-based identification and typing of many closely related bacteria, including *Pasteurellaceae*.[13] We previously used this method to differentiate among *R. pneumotropicus*, *R. heylii*, and *R. rarus*, which were known as *[P.] pneumotropica* biotypes Jawetz and Heyl and Bisgaard Taxon 17 at that time.[4]

In this current investigation, we extended the analysis of the 16S–23S ITS of the rRNA operons to *R. ratti*, *R. heidelbergensis*, and the *Rodentibacter*-related β-hemolytic taxon and compared them with the ITS of *R. pneumotropicus*, *R. heylii*, and *R. rarus*, as a basis for identification and differentiation within this group of closely related bacterial species. The length and sequence polymorphisms of the ITS allowed differentiation among the 6 species. The ability to diagnose *Rodentibacter* taxa at the species level could improve our understanding of their clinical significance.

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Table 1. Bacterial strains used in this study

Organism (n = 75)	Origin	Multiplex PCR	16S rRNA	ITS PCR profile	Reference no.
Rodentibacter heylii (n = 23)					
ATCC12555^T (type strain)	mouse	*R. heylii*	*R. heylii*	*R. heylii*	1
215/17, 220/17, 223/17a, 438/17, 439/17, 440/17, 442/17, 443/17a, 517/17, 555/17, 556/17, 557/17, 594/17, 596/17, 597/17, 33/18	mouse	*R. heylii*	*R. heylii*	*R. heylii*	7
251/17, 794/17, 1359/17, 259/18	rat	*R. heylii*	*R. heylii*	*R. heylii*	7
1026/18, 1338/18	mouse	*R. heylii*	*R. heylii*	*R. heylii*	this study
Rodentibacter pneumotropicus (n = 21)					
P421^T (CCUG12398^T; type strain)	mouse	*R. pneumotropicus*	*R. pneumotropicus*	*R. pneumotropicus*	1
212/17, 213/17, 214/17, 218/17, 223/17b, 443/17b, 518/17, 519/17, 521/17, 529/17, 530/17, 532/17, 558/17, 559/17, 560/17	mouse	*R. pneumotropicus*	*R. pneumotropicus*	*R. pneumotropicus*	7
620/19, 621/19, 646/19, 647/19, 05241	rat	*R. pneumotropicus*	*R. pneumotropicus*	*R. pneumotropicus*	this study
Rodentibacter ratti (n = 22)					
F75^T (type strain)	chicken	*Pasteurellaceae*	*R. ratti*	*R. ratti*	1
341/17, 342/17, 343/17, 345/17, 637/17, 638/17, 639/17, 1002/17, 1012/17, 1360/17, 1361/17, 257/18, 258/18, 260/18, 261/18, 262/18	rat	*Pasteurellaceae*	*R. ratti*	*R. ratti*	7
37641	mouse	*Pasteurellaceae*	*R. ratti*	*R. ratti*	this study
37811, 1739/19, 31/20, 268/20V	rat	*Pasteurellaceae*	*R. ratti*	*R. ratti*	this study
Rodentibacter rarus (n = 1)					
CCUG17206^T (type strain)	rat	*Pasteurellaceae*	*R. rarus*	*R. rarus*	1
Rodentibacter heidelbergensis (n = 5)					
Ac69^T (type strain)	rat	*Pasteurellaceae*	*R. heidelbergensis*	*R. heidelbergensis*	1
Ac71, Ac79, H199605711, H201108024	rat	*Pasteurellaceae*	*R. heidelbergensis*	*R. heidelbergensis*	1
β-hemolytic *Pasteurellaceae* (n = 3)					
1625/19	mouse	*Pasteurellaceae*	β-haem *Past.*	β-haem *Past.*	this study
1725/19, 268/20	rat	*Pasteurellaceae*	β-haem *Past.*	β-haem *Past.*	this study

Materials and Methods

Bacterial strains. In total, 75 strains including the reference strains of *R. pneumotropicus* (P421^T), *R. heylii* (ATCC12555^T), *R. rarus* (CCUG17206^T), *R. ratti* (F75^T), and *R. heidelbergensis* (Ac69^T), were used in this study. The strains were either described previously[1,7] or in the present study (Table 1). In addition to the *Rodentibacter* spp. strains mentioned, another 3 rodent β-hemolytic *Rodentibacter*-related isolates resembling the β-hemolytic isolates that had been described in previous studies[6,12] were analyzed. The bacterial strains included in the current study were identified during the routine microbiologic monitoring program of the mouse and rat colonies of the Animal Research Facility of Heinrich–Heine University Düsseldorf as described previously.[3] The health-monitoring program is part of the breeding program, which was approved by the supervisory authority under authorization number 32/12-1-5. All procedures were in accordance with the European legislation for the care and use of laboratory animals and with institutional standards.

Sample collection and preliminary identification of the clinical strains. Sterile swabs (Microbiotech, Maglie, Italy) of the nasal cavities, oropharynx, and genital mucosa of various mouse and rat strains were cultured on Columbia blood agar and MacConkey agar plates (Biomerieux, Nuertingen, Germany) for approximately 48 h at 37 °C under aerobic and anaerobic conditions. The *Pasteurellaceae*-like colonies were isolated and further characterized by PCR analysis[3] and 16S rRNA gene sequencing.[5]

ITS profiling. For ITS profiling, the primers G1 (5′ GAA GTC GTA ACA AGG 3′) and L1 (5′ CAA GGC ATC CAC CGT 3′), which are complementary to conserved regions of the 16S and 23S rRNA coding sequences flanking the ITS,[14] were used in accordance with the protocol described previously.[4] The amplification products were visualized after electrophoresis of 10 µL of the reaction mixture in a 2.8% agarose gel, which was photographed under UV exposure. A 100-bp DNA ladder (AnalytikJena, Jena, Germany) was used as a molecular size standard.

DNA sequencing and analysis. The 16S rRNA gene sequences of the strains described in this study were produced as described previously.[5] The ITS fragments of the type strains *R. ratti* and *R. heidelbergensis* and of the *Rodentibacter*-related β-hemolytic isolate 1725/19 were eluted separately from the agarose gel after electrophoresis by using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) in a bind–wash–elute procedure recommended by manufacturer. The eluted DNA samples were sequenced at the Duesseldorf University Genomics and Transcriptomics Laboratory by using the same primers as for PCR amplification. For identification, the 16S rRNA gene sequences were compared with corresponding sequences of type and reference strains by using the BLAST server (https://blast.ncbi.nlm.nih.gov/Blast.cgi).[2] Multiple-sequence alignment of ITS

sequences produced in this study and of known ITS sequences of *R. pneumotropicus*, *R. heylii*, and *R. rarus* described previously[4] was completed by using the MUSCLE alignment tool (https://www.ebi.ac.uk/Tools/msa/muscle/). The identity level was calculated by pairwise sequence alignment with the EMBOSS Needle Software (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). The ITS sequences were analyzed for the presence of tRNA genes by using tRNA-ScanE software (http://lowelab.ucsc.edu/tRNAscan-SE/).[19]

Results

Preliminary identification of *Rodentibacter* isolates. Colonies phenotypically suspected of being *Rodentibacter* were isolated from the Columbia 5% sheep blood agar plates and further classified by using the multiplex PCR assay for rodent *Pasteurellaceae* described previously[3] and by 16S rRNA gene sequence analysis (GenBank numbers LC542614 through LC542628); reference and clinical isolates described previously[1,7] were included with these strains. Based on these criteria 21 *R. pneumotropicus*, 23 *R. heylii*, one *R. rarus*, 22 *R. ratti*, 5 *R. heidelbergensis*, and 3 *Rodentibacter*-related β-hemolytic rodent *Pasteurellaceae* strains were available for ITS profiling (Table 1).

Identification of *Rodentibacter* species according to ITS profiles. PCR amplification of the ITS fragments resulted in ITS patterns shared by the reference and clinical strains, thus allowing separation of the 6 species of interest (Figure 1). All taxa included displayed one large and one small ITS fragment that differed in length between isolates. The lengths of the PCR-amplified ITS fragments of *R. pneumotropicus* (614 and 470 bp), *R. heylii* (643 and 522 bp), *R. rarus* (736 and 550 bp), *R. ratti* (633 and 472 bp), *R. heidelbergensis* (668 and 469 bp), and *Rodentibacter*-related β-hemolytic taxon (774 and 474 bp), which were determined through sequence analysis, enabled visual differentiation between the taxa included. Nevertheless, distinguishing between the patterns for *R. pneumotropicus* (Figure 1 A, lane 3) and *R. ratti* (Figure 1 A, lane 5) might be difficult. Placing the PCR products of *R. pneumotropicus* and *R. ratti* next to each other facilitates visual differentiation between the ITS profiles of these species, as shown for selected clinical strains (Figure 1 B).

Analysis of ITS sequences. The ITS sequences of *R. pneumotropicus*, *R. heylii*, and *R. rarus* reported previously[4] and of *R. ratti*, *R. heidelbergensis*, and the β-hemolytic *Pasteurellaceae* strain from the current study (GenBank numbers LC545450–LC545455) were compared through multiple sequence alignment (Figure S1).

Both ITS types demonstrated length polymorphisms between the species tested. By subtracting 53 bp from the end of 16S rRNA gene and 27 bp from the beginning of the 23S rRNA gene, the ITS sequences were 80 bp smaller than the amplified fragments described earlier. Analyses for the presence of tRNA genes revealed 2 distinct ITS for each taxa: a $ITS^{ile+ala}$ (containing the $tRNA^{Ile(GAU)}$ and $tRNA^{Ala(UGC)}$ genes) as the longer fragment and a ITS^{glu} (containing the $tRNA^{Glu(UUC)}$ gene) as the shorter. Based on all sequence analyses, the 3 tRNA genes ($tRNA^{Ile(GAU)}$, $tRNA^{Ala(UGC)}$, and $tRNA^{Glu(UUC)}$) were over 99% identical (Figure S1 A and B).

Interspecies variation of ITS. The ITS sequences from the reference strains of *R. pneumotropicus*, *R. heylii*, *R. rarus*, *R. ratti,* and *R. heidelbergensis* and the β-hemolytic *Pasteurellaceae* taxon were examined for interspecies variations. Pairwise alignment of ITS sequences revealed identity levels between species ranging from 61.8% to 85.7% for $ITS^{ile+ala}$ (Table 2) and 68.4% to 89.9% for ITS^{glu} (Table 3). Several regions with high levels of interspecies variation were identified in both ITS types (Figure S1 A and B).

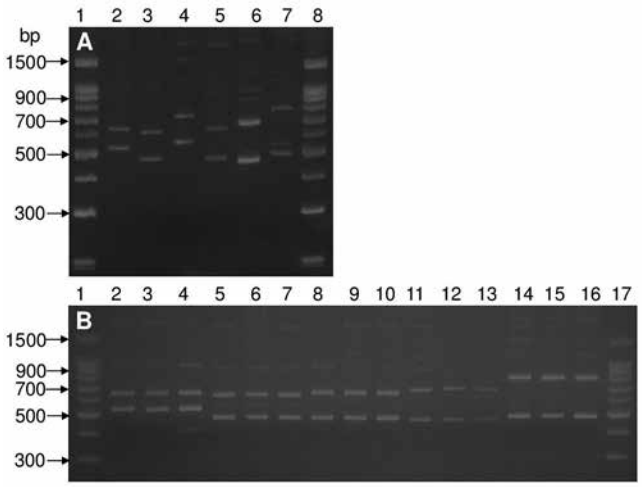

Figure 1. The ITS PCR-amplification patterns of (A) type strains and (B) clinical isolates of *Rodentibacter* spp. (A) Lanes 1 and 8, 100-bp size standards; lane 2, *R. heylii* (ATCC12555^T); lane 3, *R. pneumotropicus* (P421^T); lane 4, *R. rarus* (CCUG17206^T); lane 5, *R. ratti* (F75^T); lane 6, *R. heidelbergensis* (Ac69^T); and lane 7, β-hemolytic *Pasteurellaceae* (1725/19). (B) Lanes 1 and 17, 100-bp ladder; lanes 2 through 4, *R. heylii* (594/17, 220/17, 1338/18); lanes 5 through 7, *R. pneumotropicus* (646/19, 443/17b, 620/19); lanes 8 through 10, *R. ratti* (257/18, 260/18, 638/17); lanes 11 through 13, *R. heidelbergensis* (H201108024, Ac79, H199605711); and lanes 14 through 16, β-hemolytic *Pasteurellaceae* (1725/19, 1625/19, 268/20).

Discussion

The present investigation analyzed the 16S–23S ITS of the rRNA operons of *Rodentibacter* taxa selected as the most important for laboratory rodents. The rRNA operon is a multiple-copy gene in the prokaryotic genome. Whereas the sequences of 16S rRNA, 23S rRNA and 5S rRNA genes are the same across the multiple copies of a bacterial strain, the ITS sequences can vary in length and sequence, mainly due to the type and number of interspaced tRNA genes and their specific regulatory elements (*boxA* or *boxB*),[13] thus giving rise to multiple amplicons. Because the ITS is considered to be under less evolutionary selective pressure than are the 16S and 23S coding genes and therefore is prone to more genetic variation,[13] examination of large strain collections is expected to reveal frequent polymorphisms even in the same species. The length and sequence polymorphisms of the ITS analyzed herein showed distinct patterns for each species, thus allowing the differentiation among the *R. pneumotropicus*, *R. heylii*, *R. rarus*, *R. ratti*, *R. heidelbergensis*, and *Rodentibacter*-related β-hemolytic *Pasteurellaceae* taxon strains included in this study. However, visually differentiating between *R. pneumotropicus* and *R. ratti* based solely on ITS patterns in agarose gels is challenging.

Although limited in number, the collection of clinical isolates we analyzed was diverse and included multiple mouse and rat strains from all microbiologic units of the animal research facility of Heinrich–Heine University Düsseldorf as well as externally procured clinical and reference strains. Except for the rarely diagnosed *R. rarus* (previously known as Bisgaard Taxon 17),[9,10,] the *Rodentibacter* species included in the present study are the most prevalent *Rodentibacter* species currently found in laboratory mice and rats. The method developed here allowed for the first time the identification of *R. ratti*, *R. heidelbergensis*, and a *Rodentibacter*-related β-hemolytic *Pasteurellaceae* taxon by genetic means other than 16S rRNA gene sequencing.

After reclassification of the *[P.] pneumotropica* complex as *Rodentibacter* species, *R. heylii*, previously supposed to have mice as

Table 2. Interspecies identity (%) of $ITS^{Ile+Ala}$

	R. pneumotropicus	*R. heylii*	*R. rarus*	*R. ratti*	*R. heidelbergensis*
R. pneumotropicus					
R. heylii	78.6				
R. rarus	73.6	71.5			
R. ratti	85.7	79.2	74.3		
R. heidelbergensis	78.0	74.9	81.2	75.7	
β-hemolytic *Pasteurellaceae*	67.1	67.5	64.5	69.3	61.8

R. pneumotropicus (P421^T), *R. heylii* (ATCC12555^T), *R. rarus* (CCUG17206^T), *R. ratti* (F75^T), *R. heidelbergensis* (Ac69^T), and β-hemolytic *Pasteurellaceae* (1725/19).

Table 3. Interspecies identity (%) of ITS^{Glu}

	R. pneumotropicus	*R. heylii*	*R. rarus*	*R. ratti*	*R. heidelbergensis*
R. pneumotropicus					
R. heylii	76.4				
R. rarus	76.9	73.2			
R. ratti	89.9	76.3	75.5		
R. heidelbergensis	75.1	68.4	76.9	74.5	
β-hemolytic *Pasteurellaceae*	77.8	72.5	70.2	78.3	72.2

R. pneumotropicus (P421^T), *R. heylii* (ATCC12555^T), *R. rarus* (CCUG17206^T), *R. ratti* (F75^T), *R. heidelbergensis* (Ac69^T), and β-hemolytic *Pasteurellaceae* (1725/19).

its predominant host, was shown to also be commonly found in laboratory rats.[7] We show in the current work that *R. pneumotropicus*, another presumed mouse-predominant *Rodentibacter* species,[8] can also be found in laboratory rats. We further document that *R. ratti*, currently the most prevalent *Rodentibacter*[7,8] species with rats as its predominant host, infects laboratory mice also. Moreover, we substantiated that the β-hemolytic *Rodentibacter*-related taxon, recently found to be common among laboratory mice,[6,12] is also present in laboratory rats (Table 1).

Diagnosis of rodent *Pasteurellaceae* to the species level remains a challenging task for clinical microbiologists.[16] The current description of *Rodentibacter* spp.[1] allows better identification of members of this very diverse group of bacteria and supports increased investigation of the pathogenicity, diagnosis, and control of these infections. Nevertheless, simple diagnostic tests are not available for some *Rodentibacter* species, for which 16S rRNA gene sequencing is therefore the only option available. The present study offers a simple approach to differentiate most of the *Rodentibacter* species prevalent in laboratory rodents.

The ITS of the 6 species tested seem to be conserved among these species, given that the ITS fragments produced by PCR amplification seem similar in length among the multiple isolates tested. This similarity is consistent with the ITS of other bacterial species.[11,17,20] Conversely, the identity levels of the 2 types of ITS between the species tested were variable in length (Figure 1) and sequence (Figure S1, Tables 2 and 3). Multiple-sequence alignment revealed several variable regions, which might be exploited in the future for designing probes specific for these bacterial species.

In conclusion, ITS amplification proved to be a reliable, rapid, and easy-to-perform identification method for the most prevalent *Rodentibacter* species isolated from laboratory mice and rats. Moreover, the sequence of the ITS was conserved within a species but interspecies variation potentially can be targeted for specific identification of members of this group of bacteria. The ability to diagnose isolates to the species level could further improve understanding of the clinical significance of these bacteria.

Supplementary Material

Figure S1. Alignment of the nucleotide sequences of (A) $ITS^{ile+ala}$ and (B) ITS^{glu} (B) from the type strains of rodent *Pasteurellaceae*. Sequences corresponding to tRNA genes and *boxA* are boxed. Dashes indicate gaps introduced to improve alignment. Asterisks below the alignment indicate nucleotides identical among all of the sequences. Each sequence is labeled with the species and its length. *R. heylii* (ATCC12555^T); *R. pneumotropicus* (P421^T); *R. rarus* (CCUG17206^T); *R. ratti* (F75^T); *R. heidelbergensis* (Ac69^T); and β-hemolytic *Pasteurellaceae* (1725/19).

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