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NOTES

Phylogenetic Position of *Riemerella anatipestifer* Based on 16S rRNA Gene Sequences

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***Riemerella anatipestifer*, the causative agent of septicemia anserum exsudativa (also called new duckling disease), belongs to the family *Flavobacteriaceae* of gram-negative bacteria. We determined the DNA sequences of the *rrs* genes encoding the 16S rRNAs of four *R. anatipestifer* strains by directly sequencing PCR-amplified *rrs* genes. A sequence similarity analysis confirmed the phylogenetic position of *R. anatipestifer* in the family *Flavobacteriaceae* in rRNA superfamily V and allowed fine mapping of *R. anatipestifer* on a separate rRNA branch comprising the most closely related species, *Bergeyella zoohelcum*, as well as *Chryseobacterium balustinum*, *Chryseobacterium indologenes*, and *Chryseobacterium gleum*. The sequences of the *rrs* genes of the four *R. anatipestifer* strains varied between 0.5 and 1.0%, but all of the strains occupied the same position on the phylogenetic tree. In general, differences in *rrs* genes were observed among *R. anatipestifer* strains, even within a given serotype, as shown by restriction fragment length polymorphism of PCR-amplified *rrs* genes.**

Riemerella anatipestifer is the etiological agent of septicemia anserum exsudativa, an enzootic, contagious, often primary septicemic disease of domesticated ducklings (2, 9). The disease has a worldwide distribution, and endemic infections are restricted to commercial duck and turkey flocks (3, 18). Mortality varies from 2 to 30%, but it can be as high as 95% and is influenced by predisposing viral and bacterial infections. Thus, *R. anatipestifer* causes important losses in duck and turkey production units. *R. anatipestifer* is a gram-negative, nonmotile, non-spore-forming rod-shaped bacterium (3). So far, no particular virulence attributes have been described. Typing of *R. anatipestifer* isolates by slide and tube agglutination tests with antisera has identified 21 serotypes (12, 13).

The phylogenetic position of *R. anatipestifer* was uncertain for a long time. This organism was originally named *Pfeiferella anatipestifer* (9) and was subsequently named *Pasteurella anatipestifer* and *Moraxella anatipestifer* based more on host predilection than on conventional taxonomic criteria (5). Several phenotypic, morphological, and biochemical characteristics, including the lack of flagellation, a low DNA base ratio (29 to 35 mol% G+C), the presence of menaquinones as the sole respiratory quinones, the presence of branched-chain fatty acids at high levels, the absence of carbohydrate fermentation, and hydrolytic enzyme patterns, enabled classification of *R. anatipestifer* in the *Flavobacterium-Cytophaga* group (15), which was emended as the family *Flavobacteriaceae* (4, 19). Classification of *R. anatipestifer* in rRNA superfamily V, which contains the genera *Chryseobacterium*, *Bergeyella*, *Ornithobacterium*, *Empedobacter*, *Weeksella*, *Capnocytophaga*, *Flavobacterium*, *Cytophaga*, *Flexibacter*, and *Sphingobacterium*, was confirmed by DNA-DNA and DNA-rRNA hybridization analyses (3, 4, 17, 19). We describe in this paper the DNA sequences of the entire 16S rRNA genes (*rrs*) from four *R. anatipestifer* strains. This information allowed us to determine precisely the phylogenetic position of *R. anatipestifer* in the *Flavobacteriaceae* rRNA homology group. Also, a comparison of the sequences revealed variations among *R. anatipestifer* strains.

Amplification of 16S ribosomal DNA (rDNA). All of the strains used in this study are listed in Table 1. The type strain is *R. anatipestifer* ATCC 11845. The serotype reference strains have been described previously (12). Field strains were isolated between 1983 and 1991 from diseased ducks and were typed by performing tube agglutination tests with antisera raised against *R. anatipestifer* serotype reference strains (12); these tests were performed by workers at the Primary Production Department, Central Veterinary Laboratory, Singapore. All of the strains were grown on Columbia agar plates at 37°C in air enriched with 5% CO_2 for 24 h.

Genomic DNA was extracted by the rapid guanidium thiocyanate method of Pitcher et al. (14). PCRs were carried out in 50-μl reaction mixtures containing 5 μl of 10× PCR buffer supplemented with $MgSO_4$ (Boehringer, Mannheim, Germany), 20 pmol of each primer, 1 mM deoxynucleotide triphosphate (Boehringer), 2.5 U of *Pwo* DNA polymerase (Boehringer), and 100 ng of genomic DNA. PCR amplifications were performed with a GeneAmp PCR System model 2400 DNA thermal cycler (Perkin-Elmer) as follows: 35 cycles consisting of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min, with a final extension step consisting of 7 min at 72°C to allow all of the extension products to be completed. The primers used for PCR generation of *rrs* gene fragments were universal primers 16SUNI-L and 16SUNI-R (11) derived from the *Escherichia coli rrs* sequence (6). Negative controls that contained all of the components described above except the template DNA were included in each experiment. The PCR products were examined by electrophoresis of small aliquots in a 0.7% agarose gel at 110 V for 2 h. For further analysis the remainder of each PCR product was purified with a QIAquick PCR purification

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TABLE 1. *R. anatipestifer* strains

Strain[a]	Serotype
ATCC 11845^T	ND[b]
35/90	1
179/90	1
205/90	1
105/91	1
17/91	2
389/89[c]	6
203/89	7
232/89	10
2/91	10
76/91	11
84/91	11
134/90	13
664/83[c]	14
743/85[c]	15
204/88	15
110/89	15
135/90	15
977/83[c]	17
540/86[c]	18
30/90[c]	19
53/91	19
59/91	19

[a] ATCC, American Type Culture Collection. All strains that were not type strains or serotype reference strains were field isolates of the Central Veterinary Laboratory, Singapore.

[b] ND, not determined.

[c] Serotype reference strain (12).

TABLE 2. Accession numbers of 16S rRNA sequences of *R. anatipestifer* strains and related bacteria

Organism	GenBank accession no.
Riemerella anatipestifer ATCC 11845^T	U60101[a]
Riemerella anatipestifer serotype 6 reference strain	U60102[a]
Riemerella anatipestifer serotype 15 reference strain	U60103[a]
Riemerella anatipestifer serotype 19 reference strain	U60104[a]
Chryseobacterium gleum	M58772
Chryseobacterium indologenes	M58773
Chryseobacterium balustinum	M58771
Chryseobacterium meningosepticum	M58776
Empedobacter brevis	M59052
Ornithobacterium rhinotracheale	L19156
Porphyromonas catoniae	X82823
Cytophaga lytica	M62796
Rikenella microfusus	L16498
Bergeyella zoohelcum	M93153
Weeksella virosa	M93152
Capnocytophaga ochracea	U41350
Flavobacterium columnare	M58781
Flectobacillus glomeratus	M58775
Blattabacterium sp.	Z35666
Bacteroides putredenis	L16497

[a] Determined in this study.

kit (Qiagen, Hilden, Germany) used according to the manufacturer's instructions.

Sequencing and phylogenetic analysis. Purified PCR amplification products of the 16S rRNA genes of type strain ATCC 11845 and the serotype 6, 15, and 19 reference strains were completely sequenced directly on both strands by using an AmpliTaq FS dye terminator kit (Perkin-Elmer) and oligonucleotide primers described previously (11). The primers were derived from the conserved regions of the 16S rRNA genes of many gram-negative and gram-positive bacterial species and were designed to allow complete sequencing of *rrs* genes on both strands in one round. Each sequencing reaction was performed with approximately 50 ng of template DNA per reaction mixture and 5 pmol of primer. The sequences of the products were determined with an ABI Prism model 310 genetic analyzer and were edited and assembled by using the Sequencher 3.0 program (GeneCodes, Ann Arbor, Mich.) to obtain entire sequences. Alignment of the ATCC 11845^T (T = type strain) *rrs* gene sequence with EMBL and GenBank DNA and RNA sequence databank data by using the program BLASTN (1) revealed that the phylogenetically most closely related bacterial species all belonged to the *Flavobacteriaceae* rRNA homology group. Fourteen most closely related 16S rRNA sequences and two more distantly related sequences were chosen for the phylogenetic analysis (Table 2). Each of the four *R. anatipestifer rrs* sequences was compared with the sequences of these 16 reference organisms. The evolutionary distances between sequences were computed by using the algorithm of Jukes and Cantor (10) with the Mega 1.02 phylogenetic analysis software package. Ambiguous nucleotides which were present in some sequences in the database were not considered in the comparison. A tree to estimate the phylogenetic relationships among the organisms was derived by using the neighbor-joining method (16) (Fig. 1). The topology of the distance tree was tested by performing 1,000 bootstrap resamplings of the data. Our analysis of the 16S rRNA gene sequences of four different strains of *R. anatipestifer* confirmed the phylogenetic position of *R. anatipestifer* within rRNA superfamily V in the taxonomic neighborhood of the genera *Chryseobacterium*, *Bergeyella*, and *Ornithobacterium*. The four *R. anatipestifer* strains occupied the same position on the phylogenetic tree (Fig. 1) generated in this study, which is branched like the tree based on DNA-rRNA hybridization analysis results (17). The organism that is most closely related to *R. anatipestifer*, *Bergeyella zoohelcum*, has 92.8% homolo-

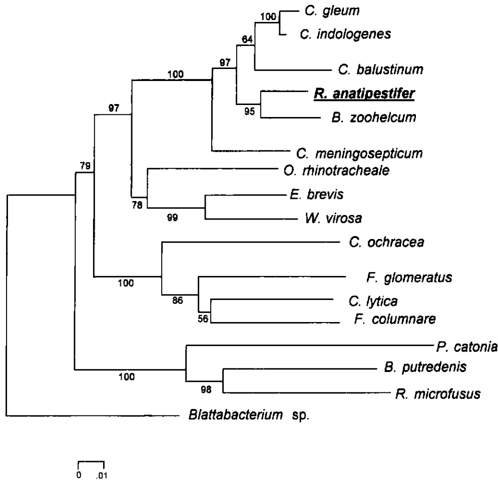

FIG. 1. Phylogenetic tree based on 16S rRNA gene sequence similarity values for *R. anatipestifer* type strain ATCC 11845 and 14 closely related species, as well as the more distantly related organisms *Bacteroides putredenis* and *Blattabacterium* sp. Phylogenetic relationships were established with the Mega 1.02 program; corrected distances were calculated with the Jukes-Cantor algorithm, and a tree was derived by using the neighbor-joining method. Bootstrap values were calculated from 1,000 trees. Scale bar = 1% sequence divergence. The *R. anatipestifer* reference strains for serotypes 6, 15, and 19 occupy the same phylogenetic position as the type strain despite the heterogeneity detected in the 16S rRNA genes. See Table 2 for spelled-out names.

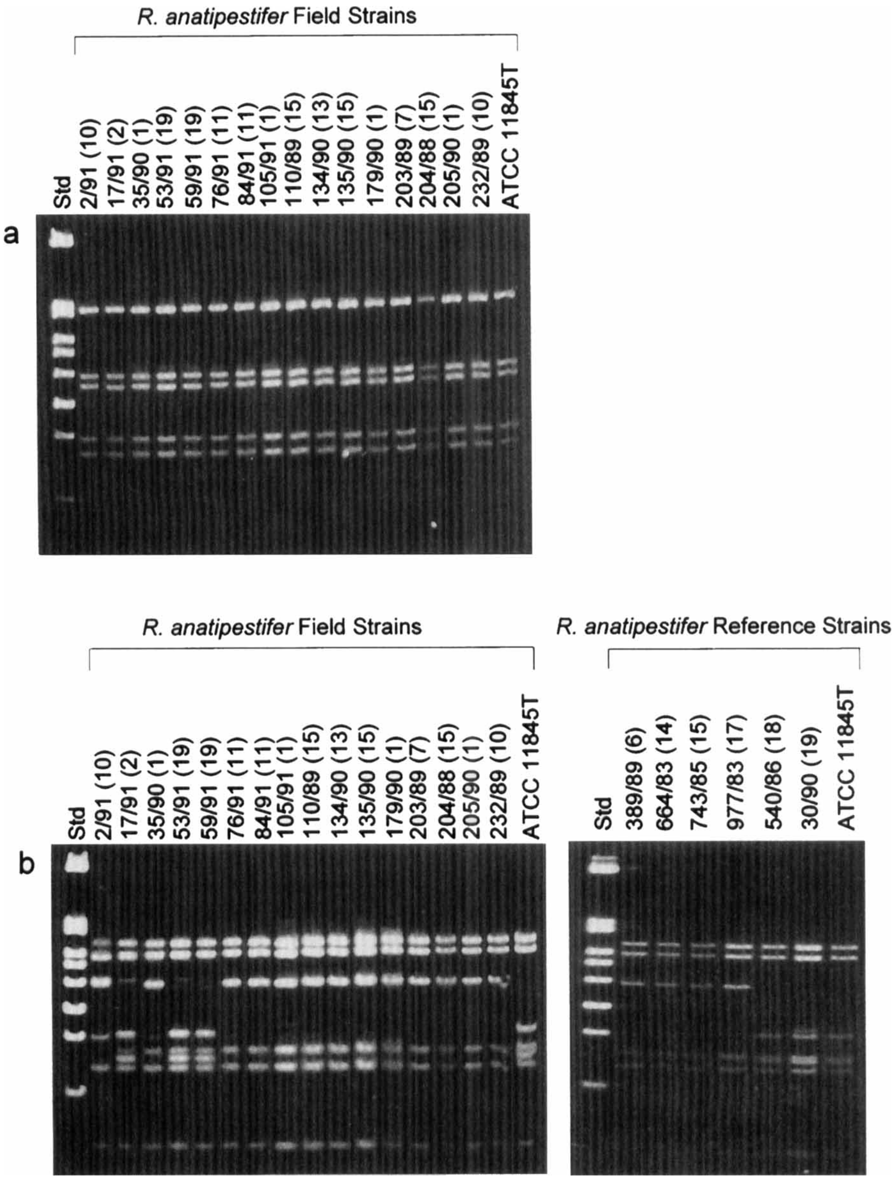

FIG. 2. RFLP analysis of *R. anatipestifer* strains. Chromosomal DNAs of serotype reference strains, the type strain, and field strains were extracted and used as templates for PCR amplification of 16S rRNA genes with universal primers. The PCR products were digested with enzymes, and the fragments were electrophoresed on 8% polyacrylamide gels. Bands were visualized by ethidium bromide staining. The standards used were *Hin*fI-digested pBR322 fragments. (a) *Hae*III digests, showing identical RFLP patterns. (b) *Dde*I digests, showing minor heterogeneity in RFLP patterns among the strains. The numbers in parentheses indicate serotypes. Std, standard.

gous nucleotides in its 16S rRNA sequence. Other closely related organisms are *Chryseobacterium gleum*, *Chryseobacterium indologenes*, *Chryseobacterium balustinum*, and *Chryseobacterium meningosepticum*, which exhibit 91.4 to 92.6% sequence homology in the 16S rRNA genes. *R. anatipestifer* and *B. zoohelcum* each constitute a separate rRNA branch, while *C. gleum*, *C. indologenes*, and *C. balustinum* constitute another branch. *Chryseobacterium meningosepticum* occupies a separate branch.

Based on DNA-rRNA hybridization studies (17), *R. anatipestifer* was shown to belong to the family *Flavobacteriaceae*. *R. anatipestifer*, however, differs phenotypically from the genera *Chryseobacterium* and *Bergeyella*, which also belong to the *Flavobacteriaceae* rRNA homology group, by its capnophilic metabolism, by the absence of pigments, and by its fatty acid and protein contents (17). Our sequence data confirm the phylogenetic results obtained previously in DNA-rRNA hybridization studies (17). Moreover, our data allow fine-tuned classification of *R. anatipestifer* and affirm previous phenotypic and genotypic conclusions which support a separate genus for this taxon.

Alignment of the *rrs* sequences of the four *R. anatipestifer* strains examined (strain ATCC 11845^T and the reference strains of serotypes 6, 15, and 19) revealed base differences at 14 positions. Of the 14 base differences, 3 (at positions 559, 747, and 1190) were determined to be in conserved regions of the 16S rRNA gene of the family *Flavobacteriaceae*. Despite being in the conserved regions, the bases at two of the positions, positions 559 and 1190, seemed to be variable in members of the *Flavobacteriaceae*.

RFLP. In order to obtain a broader view of the variability in the *rrs* genes of *R. anatipestifer*, a restriction fragment length polymorphism (RFLP) analysis was performed with type strain ATCC 11845, serotype reference strains, and field isolates. Aliquots (3 μl) of PCR products from the *R. anatipestifer* strains were digested with *Rsa*I, *Alu*I, *Hin*fI, *Hae*III, and *Dde*I. The products were examined on an 8% acrylamide gel electrophoresed with TBE buffer (0.4 M Tris, 0.4 M boric acid, 0.01 M EDTA; pH 8.3) for 4 h at 80 V. The digested fragments were visualized by staining with ethidium bromide. The five enzymes were chosen on the basis of the *rrs* sequence of *R. anatipestifer* ATCC 11845^T.

The restriction fragment patterns obtained with *Rsa*I-, *Alu*I-, *Hin*fI-, and *Hae*III-digested 16S rDNA PCR products were the same for all of the strains analyzed. The *Hae*III restriction profiles are shown in Fig. 2a. Minor heterogeneity among the strains was observed in the restriction fragment profiles obtained with *Dde*I (Fig. 2b), which apparently divided the strains analyzed into two groups. This grouping of strains, however, seemed not to be useful. Strains 35/90 and 2/91, members of serotypes 1 and 10, respectively, have *Dde*I restriction profiles that are different from those of other strains belonging to these two serotypes (Fig. 2b). Therefore, *Dde*I profiles are not consistent with serotyping. In addition, the profiles were only dependent on a small number of significant bases in the 16S rRNA gene (five restriction enzyme recognition sites, only two of which were located in the variable regions).

Alignments of *R. anatipestifer rrs* sequences revealed eight base differences between ATCC 11845^T and serotype 19 and two base differences between serotypes 6 and 15, which have the same *Dde*I restriction fragment profiles. Ten base differences were observed between ATCC 11845^T and serotype 15, which have different *Dde*I restriction fragment profiles. This confirms that *Dde*I profiles do not distinguish different subtypes of *R. anatipestifer.*

Nucleotide differences in the 1% range were determined for the 16S rRNA genes of the four *R. anatipestifer* strains analyzed in this study. In addition, the results of the restriction fragment analysis of the 16S rDNAs of *R. anatipestifer* strains revealed differences within a given serotype (Fig. 2b). These observations are not surprising as high levels of intraspecific variation (within and between strains) have been reported in the 16S rRNA sequences of prokaryotes in the GenBank database (7). It has been suggested that organisms with less than 15 base differences in the 16S rRNA gene generally belong to the same species (8), a suggestion which seems to apply to *R. anatipestifer.*

The results obtained in this study are important for basic approaches to molecular analysis in *R. anatipestifer* since several functions and attributes are expected to vary among different strains, even within the same serotype. This should be particularly significant for future development of genetic methods for subtyping *R. anatipestifer* strains in order to avoid confusion due to subclassifications that are not congruent with serotypes.

Nucleotide sequence accession numbers. The sequence data are accessible under the following EMBL and GenBank accession numbers: ATCC 11845^T, U60101; serotype 6 reference strain 389/89, U60102; serotype 15 reference strain 743/85, U60103; and serotype 19 reference strain 30/90, U60104.

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