Comparison of the 16S Ribosomal DNA Sequences from the Intracellular Agents of Proliferative Enteritis in a Hamster, Deer, and Ostrich with the Sequence of a Porcine Isolate of *Lawsonia intracellularis*

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Proliferative enteritis is an enteric disease that affects a variety of animals. The causative agent in swine has been determined to be an obligate intracellular bacterium, *Lawsonia intracellularis*, related to the sulfate-reducing bacterium *Desulfovibrio desulfuricans*. The intracellular agents found in the lesions of different animal species are antigenically similar. In addition, strains from the pig, ferret, and hamster have been shown to be genetically similar. In this study we performed a partial 16S ribosomal DNA sequence analysis on the intracellular agent of proliferative enteritis from a hamster, a deer, and an ostrich. These sequences to that of the porcine *L. intracellularis* isolate. Results of this study indicate that the intracellular agents from these species with proliferative enteritis have high sequence similarity, indicating that they are all in the genus *Lawsonia* and that they may also be the same species, *L. intracellularis*.

The intracellular agents of PE are morphologically identical in all species that the disease affects (4, 5, 17, 27, 34, 36). The ability of monoclonal antibodies developed from a pig isolate of the organism to detect the organism in tissues from other species demonstrates that the intracellular organisms seen in lesions from different species are antigenically similar (4, 6, 14, 24, 34, 36). Sequence analysis of the 16S rDNA from this organism from the pig (7, 21), the hamster (6, 26), and the ferret (6) suggests that the intracellular agents of PE in these species are also genetically similar.

In this study we performed a complete 16S rDNA sequence analysis of the intracellular agent of PE in the hamster and a partial 16S rDNA sequence analysis on the intracellular agent of PE extracted from formalin-fixed, paraffin-embedded tissues from a deer and an ostrich with PE. The sequence obtained demonstrated high similarity between the 16S rDNA from the intracellular agent of PE from these species and that of the pig isolate of *L. intracellularis*. These results suggest that the intracellular agents of PE in the hamster, deer, and ostrich are all isolates of *L. intracellularis*.

**MATERIALS AND METHODS**

**Samples.** The hamster intracellular agent of PE (strain auratus) was grown in INT 407 human embryonic intestinal epithelial cells, and the DNA was extracted for PCR and sequence analysis. The organism was fed to newly weaned hamsters (Mesocricetus auratus) according to protocols previously described (31). The animals were necropsied at 21 days, and the ilea were harvested. Ilea from one of these experimentally infected hamsters and from a deer (Odocoileus virginianus) and an ostrich (Struthio camelus) with spontaneous PE were examined. PE was diagnosed by histopathologic examination of formalin-fixed, paraffin-embedded tissues stained with Warthin-Starry silver stain and by multiplex PCR (2). The hamster organism was kindly provided by Harold Stills, College of Veterinary Medicine, Ohio State University, Columbus. Deer tissues were kindly provided by Richard DrolcT, Universite de Montreal, Quebec, Canada. Ostrich tissues were kindly provided by Leslie Woods, Veterinary Diagnostic Laboratory, University of California, Davis.) Tissues frozen at −70°C or formalin-fixed, paraffin-embedded samples of the ilea from these animals were selected for DNA extraction, amplification, cloning, and sequencing.

**Sample digestion.** Formalin-fixed, paraffin-embedded samples were digested by methods similar to the procedures of Isola et al. (11). Briefly, 10 to 20 5-mm-thick slices were cut from each block, deparaffinized once in 1 ml of xylene, and rinsed twice in 100% ethanol. Samples were dried at room temperature and resuspended in 1 ml of DNA extraction buffer (0.3 mg of proteinase K/ml, 100
DNA extraction. DNA was extracted from cultured cells with a phenol-chloroform extraction (28). DNA was extracted from tissue samples with diatomaceous earth and guanidine thiocyanate (GuSCN) as described by Boom et al. (1). Briefly, 0.5 ml of a sample was mixed with 0.9 ml of lysis buffer (6 M GuSCN, 0.1 M Tris-HCl [pH 6.4], 0.65% Triton X-100) and 40 μl of diatomaceous-earth suspension (200 mg/ml of 0.17 M HCl). The sample was vortexed, incubated at 20°C for 15 min, and pelleted at 14,000 × g for 2 min. A GuSCN rinse (6 M GuSCN, 0.1 M Tris-HCl [pH 6.4]) and twice in 200 μl of 70% ethanol, with vortexing and pelleting of the sample with each rinse. The pellet was dried at 20°C overnight and resuspended in 100 μl of PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂). The samples were incubated at 50°C for 10 min, vortexed, and pelleted at 14,000 × g for 2 min, and the supernatant was saved for PCR.

PCR primers. Primers used for PCR amplification of the 16S rDNA of the hamster PE have been described (23). The primer and previously used to sequence the 16S rDNA sequence of L. intracellularis (7, 21). Each pair is capable of initiating amplification of nearly full-length 16S rDNA (1.6 kb) from a taxonomically wide range of bacteria. Extension primers were developed from internal sequences as needed (21). The 16S rDNA sequence analysis of the intracellular organism from the deer and ostrich were derived from the 16S rDNA sequence of the L. intracellularis type strain, NCTC 12656 (National Collection of Type Cultures, Colindale, London, England), purified from the leaf mucosa of a pig with PE (7). The samples of the PCR products were submitted to the Microchemical Facility, Institute for Human Genetics, University of Minnesota, for automated sequencing analysis (model 573 fluorimeter) and previously used to sequence the L. intracellularis (7, 21). Each pair is capable of initiating amplification of nearly full-length 16S rDNA (1.6 kb) from a taxonomically wide range of bacteria. Extension primers were developed from internal sequences as needed. The 16S rDNA sequence analysis of the intracellular organism from the deer and ostrich were derived from the 16S rDNA sequence of the L. intracellularis type strain, NCTC 12656, the swine intracellular agent of PE (GenBank accession no. U30147), with that for the intracellular agent of ferret proliferative colitis (GenBank accession no. U07570), and with another isolate of the intracellular agent of hamster PE (GenBank accession no. U07569), and with another isolate of the intracellular agent of hamster PE (GenBank accession no. U084623) (26). To also determine the relationships between these organisms and their nearest taxonomic relative with pathogenic potential, L. intracellularis was compared with a Desulfovibrio isolate from a human liver abscess (GenBank accession no. U42221) (32) by the same methods.

Nucleotide sequence accession numbers. The sequences of the cloned 16S rDNAs from each species have been submitted to GenBank with the following accession numbers: hamster, U65995; deer, U65996; ostrich, U65997.

RESULTS

Diagnosis of PE. Ileum from a hamster, an ostrich, and a deer, all with PE, were fixed, stained with hematoxylin and eosin and with Warthin-Starry silver stains, and examined by light microscopy. There was hyperplasia of the ileal crypt epithelium with silver-stained organisms present within the apical cytoplasm of crypt enterocytes in the hamster, deer, and ostrich with PE (Fig. 1).

Sequence analysis. Individual recombinant clones, each containing the partial 16S rDNA sequence from the intracellular agent of PE from the hamster, the ostrich, or the deer, were selected for plasmid purification and cycle sequencing with fluorescence-labeled nucleotides and automated sequence determination. These sequences were compared with the sequence of the L. intracellularis type strain (an isolate of ileal symbiont intracellularis, NCTC 12656, grown in cell culture; GenBank accession no. U30147) (21) and with sequences determined for strains of the intracellular agent of proliferative colitis in the ferret and of the agent of PE in the hamster. A 1,109-bp sequence corresponding to bp 358 to 1465 of L. intracellularis was determined from the hamster sample. Sequences of 255-bp corresponding to bp 815 to 1070 of L. intracellularis were determined from the ostrich and deer samples. There was 100% sequence identity between the 255-bp sequence from L. intracellularis and those of the intracellular agents of PE in the hamster, deer, and ostrich (Fig. 2). There was 100% similarity over the entire 1,109-bp sequence from the hamster and the corresponding sequence from L. intracellularis (data not shown). The sequences of the cloned 16S rDNAs from each species have been submitted to GenBank (see above for accession numbers). The sequences were 100% similar to the sequence of the ferret intracellular organism over this region. Our hamster sequence had 98.4% similarity over the entire 16S rDNA with another sequence (GenBank accession no. U06423) (26), of the same strain (aursatus) but only 97.7% similarity with the partial sequences we determined for the deer and ostrich isolates. Another isolate of the intracellular agent of hamster PE (GenBank accession no. U07569) (6) had 98.8% 16S rDNA sequence similarity with our hamster isolate over a partial sequence that did not include the hyper-variable region. L. intracellularis had 91% 16S rDNA sequence
FIG. 1. Intestinal crypts from a hamster (A), a deer (B), and an ostrich (C) with PE stained with Warthin-Starry silver stain. Numerous elongate, curved organisms are present within the apical cytoplasm of the crypt enterocytes (arrows). Magnification, ×660.

FIG. 2. DNA sequences of a portion of the 16S rDNA of the intracellular agents of PE derived from the hamster (strain auratus), deer (strain 93-3669), and ostrich (strain D94-06196) compared to the consensus sequences of a pig isolate of L. intracellularis (NCTC 12056; GenBank accession no. U30147) and of D. desulfuricans (GenBank accession no. M34113), the closest relative of L. intracellularis. Underlined regions correspond to 878F, the forward primer for the PCR amplification of the intracellular organism 16S rDNA from lesions of PE to 1100R, the reverse primer, and to a hypervariable region that distinguishes L. intracellularis from D. desulfuricans by nine bases. Dashes indicate gaps introduced to facilitate comparisons.

similarity with a Desulfovibrio isolate from a human liver abscess.

**DISCUSSION**

The results of this study show that the 16S rDNA sequences of the intracellular agents of PE from the pig, hamster, deer, and ostrich have high similarity, suggesting that they are all isolates of *L. intracellularis*. rDNA sequence analysis is a powerful technique that is used to identify the taxonomic position of prokaryotes (35). Partial 16S rDNA analysis has been used to determine the relationships between species of *Desulfovibrio* (3), the genus most closely related to *L. intracellularis* (7, 21). *D. desulfuricans* and *L. intracellularis* have 91% 16S rDNA sequence similarity (7). The region of the 16S rDNA of the intracellular agents of PE from the hamster, deer, and ostrich that was sequenced includes a hypervariable region that differentiates *L. intracellularis* from *D. desulfuricans*, its closest relative (Fig. 2) (7). There is 96.1% similarity between *L. intracellularis* and *D. desulfuricans* across this region. There was 100% sequence similarity across this hypervariable region (and for the entire partial 16S rDNA sequence we determined) between *L. intracellularis* and the intracellular agents of PE in the hamster, deer, and ostrich.

These results closely parallel those of other investigators who have shown that the intracellular agents of proliferative colitis in the ferret (6) and of PE in the hamster (6, 26) are closely related to *L. intracellularis*, based on 16S rDNA sequence similarity. We obtained a slightly different sequence for the same strain (auratus, isolated from a hamster) from that of Peace et al. (26), with only 98.4% similarity overall and 97.7% over the partial sequence we determined for the deer and
ostrich isolates. A hamster isolate sequence obtained by Fox et al. (6) was 98.8% similar to our sequence overall; it did not include the region with the partial sequence we determined for the ferret, pig, deer, and ostrich isolates. However, based on the fact that other isolates of the intracellular agent of PE from disparate animal species, such as the ferret, pig, deer, and ostrich, have 100% sequence similarity across the partial sequence we determined, it is likely that the differences between the hamster isolates represent sequencing errors rather than strain diversity. Different isolates of L. intracellularis from pigs have not shown great diversity in the 16s rDNA sequences (21). Nevertheless, these similarities are high, making it likely that all of these isolates are members of the genus Lawsonia, if not the same species, L. intracellularis. DNA-DNA reassociation data would be needed to determine if organisms with such high 16s rDNA similarities are the same species (30). However, it is not possible to perform these experiments with these organisms, because they cannot be grown on cell-free media.

The organisms in the genus Lawsonia are clearly different from D. desulfuricans ATCC 27774 (the type strain) (7) and from a Desulfovibrio isolate from a human liver abscess (32), based on only 91% 16s rDNA sequence similarity. In addition to the differences in 16s rDNA sequences, these organisms differ from the Desulfovibrios in several other important areas. They are obligate intracellular parasites (7, 15, 21, 31), whereas the Desulfovibrio species can be grown on cell-free media (3, 32). In addition, the presence of the intracellular organism is always associated with lesions, and the pathogenesis of the disease (4–7, 17, 23, 24, 27, 31) is clearly distinct from the diseases that have been associated with the normally nonpathogenic Desulfovibrio (32).

We have previously discussed the amplification of L. intracellularis DNA from formalin-fixed, paraffin-embedded tissues by PCR (2). It was necessary to use prolonged digestion with proteinase K and subsequent extraction with diatomaceous earth in the presence of GuSCN to purify DNA for successful amplification from these samples. While the reason for this is not known, we hypothesize that either there was very little target DNA, the target DNA was extensively cross-linked by formalin fixation and inaccessible to the DNA polymerase, the bacterial cell wall was resistant to degradation, or there were PCR inhibitors present in the fixation preparation. Preparation of human or viral nucleic acids from archival tissues for PCR amplification does not require prolonged digestion (11, 29), while preparation of bacterial DNA may (25).

This study demonstrates that the causative agent of PE in pigs, L. intracellularis, is closely related or identical to the intracellular agents of PE in the hamster, deer, and ostrich. PE is emerging as a worldwide disease that occurs in many species of animals. The causative agent, L. intracellularis, appears to be a versatile pathogen with a wide host range. An understanding of the relationships between different isolates of this organism will greatly facilitate the study of the epidemiology of the disease.

REFERENCES