Phylogenetic Positions of *Clostridium chauvoei* and *Clostridium septicum* Based on 16S rRNA Gene Sequences

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The sequences of the 16S rRNA genes (rrs genes) of *Clostridium chauvoei*, the causative agent of blackleg in cattle, and the phenotypically related organism *Clostridium septicum* were determined. After amplification of 1,507-bp PCR fragments from the corresponding rrs genes, the sequences were determined in a single round of sequencing by using conserved primer regions. A sequence similarity analysis of the sequences revealed the close phylogenetic relationship of *C. chauvoei* and *C. septicum* in *Clostridium* cluster I (M. D. Collins, P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cal, H. Hippel, and J. A. E. Farrow, Int. J. Syst. Bacteriol. 44:812–826, 1994), which includes *Clostridium carnis*, *Clostridium perfringens*, *Clostridium botulinum*, and *Clostridium tetani*. We found that 99.3% of the nucleotides in the genes of *C. chauvoei* and *C. septicum* are identical.

*Clostridium chauvoei*, the etiologic agent of blackleg, is a very virulent pathogen of cattle that causes fever, depression, lameness, and a high level of mortality. Because of the similar courses of the diseases, blackleg was formerly frequently confused with malignant edema caused by *Clostridium novyi* and with anthrax caused by *Bacillus anthracis*. Symptoms similar to blackleg symptoms can be caused by *Clostridium septicum*, *Clostridium perfringens*, and *Clostridium sordellii* (1). *C. chauvoei* is a gram-positive, anaerobic, spore-forming bacterium that has strong hemolytic activity. The genus *Clostridium* consists of organisms whose G+C contents vary from 22 to 55 mol% (5). Many newly identified species have been described recently; the genus now contains about 100 species, some of which are pathogenic and some of which are nonpathogenic (8). Many pathogenic clostridia, including *C. chauvoei*, have been shown to produce toxins which seem to be responsible for pathogenicity (for a review see reference 4). Despite the extensive work on the phylogeny of the species belonging to the genus *Clostridium* by Collins et al. (3), the phylogenetic positions of *C. chauvoei* and *C. septicum* have never been determined. Collins et al. (3) sequenced the rrs genes encoding the 16S rRNAs of 34 different clostridial species and determined their phylogenetic relationships to more than 80 previously determined clostridial rrs sequences and rrs sequences of representative species of other low-G+C-content gram-positive genera. Almost one-half of the clostridial species examined belonged to a phenotypically well-defined cluster designated cluster I. The remaining clostridial species exhibited considerable degrees of phylogenetic diversity and formed 19 clusters and four individual lines of descent.

In order to resolve the phylogenetic position of *C. chauvoei*, we determined its complete rrs gene sequence from DNA obtained by PCR amplification of genomic DNA from the *C. chauvoei* type strain and a field isolate. In parallel, we also determined the rrs gene sequence of the phenotypically very similar organism *C. septicum*.

**Amplification of 16S ribosomal DNA.** The strains used in this study were *C. chauvoei* ATCC 10092 T (T = type strain) and diagnostic field isolates of *C. chauvoei*, as well as *C. septicum* ATCC 12646 T and ATCC 8065. All strains were grown in Schaedler broth (code no. CM 497; Oxoid, Basingstoke, United Kingdom) anaerobically at 37°C without shaking. Chromosomal DNA was prepared by the guanidinium thiocyanate method of Pitcher et al. (7). PCR were performed with a model PE9600 automated thermal cycler equipped with MicroAmp tubes (Perkin-Elmer Cetus, Norwalk, Conn.) by using a polymerase with proof-reading activity in order to avoid artificial sequence divergence. Directly sequencing the PCR product without further cloning increased the accuracy of the sequence determination. Each 50-μl reaction mixture contained 5 μl of 10× PCR buffer, 20 pmol of each primer, 1 mM deoxynucleoside triphosphate, 2.5 U of Pwo DNA polymerase (Boehringer, Mannheim, Germany), and 100 ng of genomic DNA. The PCR conditions were as follows: 35 cycles consisting of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s. A final extension step for 7 min at 72°C was also included. The primers used for PCR generation of rrs gene fragments were universal primer 16SUNI-L (Table 1) derived from the *Escherichia coli* rrs sequence (2) and primer UNI16S-R (Table 1) derived from the rrs sequence of *Clostridium botulinum* (6), which was used to amplify the most 3′ part of the *C. chauvoei C. septicum rrs* genes.

**Sequencing.** The PCR product was purified with a QiAquick PCR purification kit used according to the recommendations.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SRNAI-S</td>
<td>5′ CTACGGAGGAGCAGCAGTGGG 3′</td>
<td>341–361</td>
</tr>
<tr>
<td>16SRNAI-S</td>
<td>5′ GTGTGAAGCTGGATATGCTGA 3′</td>
<td>682–702</td>
</tr>
<tr>
<td>16SRNIII-S</td>
<td>5′ GCCTGAGGAGTACCGGCAAG 3′</td>
<td>880–901</td>
</tr>
<tr>
<td>16SRNIV-S</td>
<td>5′ GGTTAAGTCCCGCAACGAGCGC 3′</td>
<td>1087–1108</td>
</tr>
<tr>
<td>16SRNAV-S</td>
<td>5′ CCCACTGTTGCGCTCCTCGTAG 3′</td>
<td>361–341</td>
</tr>
<tr>
<td>16SRNAV-S</td>
<td>5′ CTACCGATTCTACGCGCTAC 3′</td>
<td>702–682</td>
</tr>
<tr>
<td>16SRNVIII-S</td>
<td>5′ CTCTCGGAGGTCCGATCCGACG 3′</td>
<td>901–880</td>
</tr>
<tr>
<td>16SRNAVIII-S</td>
<td>5′ GCCGTGGAGGGACGATCCAACC 3′</td>
<td>1108–1087</td>
</tr>
<tr>
<td>16SRNI-L</td>
<td>5′ AGAATTTATGATCAGCCTAC 3′</td>
<td>5–27</td>
</tr>
<tr>
<td>16SRNI-R</td>
<td>5′ GTTGTAACTGGAGGGTGTTGAC 3′</td>
<td>1410–1391</td>
</tr>
<tr>
<td>UNI16S-R</td>
<td>5′ AAGGAGGTAGTATCCAGCCGCA 3′</td>
<td>1540–1521</td>
</tr>
</tbody>
</table>

* The numbers indicate the positions relative to the *E. coli* rrs sequence (accession number J01859).

* All of the primers except UNI16S-R, which was obtained from the *C. botulinum* sequence, are universal to clostridia and *E. coli*, as determined by rrs comparisons.

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The sequences of strain ATCC 10092T and the field isolate were identical. Likewise, the sequences of C. botulinum A and B, C. botulinum type A and B, C. botulinum type C, C. sporogenes, Clostridium tetani and C. perfringens were used, we found that on the basis of their rrs sequences C. chauvoei and C. septicum are phylogenetically located near C. perfringens. For fine phylogenetic positioning of C. chauvoei and C. septicum we included strains that are most closely related to C. perfringens in our final analysis, as well as the cluster II organism C. histolyticum as an outgroup. This comparison revealed that C. chauvoei and C. septicum are closely related to Clostridium carnis (Fig. 1). We found that the level of rrs sequence similarity was 97% between C. chauvoei and C. carnis, as well as between C. septicum and C. carnis.

The close phylogenetic relationship between C. chauvoei and C. septicum is a molecular reflection of the phenotypic similarity of these organisms. C. chauvoei is relatively difficult to distinguish from C. septicum, which might create problems in diagnosis, since C. septicum can cause symptoms very similar to blackleg symptoms. In this respect we hope to develop a specific identification system for C. chauvoei based on the rrs gene sequences presented in this paper. This could be done with PCR and/or restriction fragment length polymorphism, whereas there might be some limitations because of the few nucleotide differences. It might even be possible to sequence specific parts of the rrs gene in order to determine by sequence analysis the correct species isolated from clinical material. In this way our work should help improve diagnostic tools for C. chauvoei based on molecular genetics.

**Nucleotide sequence accession numbers.** The sequences which we determined have been deposited in the GenBank database under accession numbers U51843 (C. chauvoei) and U59278 (C. septicum).

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REFERENCES

declaration of interests.

prokaryotes, 2nd ed. Springer-Verlag, New York.
saccharolytic Clostridium botulinum types B, E and F and related clostridia as revealed by
Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The
prokaryotes, 2nd ed. Springer-Verlag, New York.