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

PETER KUHNERT,* SELJA E. CAPAUL, JACQUES NICOLET, AND JOACHIM FREY

Institute of Veterinary Bacteriology, University of Bern, CH-3012 Bern, Switzerland

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 region primers. 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. Cai, H. Hippe, 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*, *C. novyi*, *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 new *Clostridium* 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. sep-*

ticum ATCC 12464^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 1. Primers used for amplification and/or sequencing of *rrs* DNA

Primer	Sequence	Location ^a
16SRNAI-S	5' CTACGGGAGGCAGCAGTGGGG 3'	341–361
16SRNAII-S	5' GTGTAGCGGTGAAATGCGTAG 3'	682–702
16SRNSIII-S	5' GCCTGGGGAGTACGGTGCAG 3'	880–901
16SRNAIV-S	5' GGTAAAGTCCCGCAACGAGCGC 3'	1087–1108
16SRNAV-S	5' CCCCACTGCTGCCTCCCGTAG 3'	361–341
16SRNAVI-S	5' CTACGCATTTCACCGTACAC 3'	702–682
16SRNSVII-S	5' CTTGCGACCGTACTCCCCAGGC 3'	901–880
16SRNAVIII-S	5' GCGCTCGTTGCGGGACTTAACC 3'	1108–1087
16SUNI-L	5' AGAGTTTGTATCATGGCTCAG 3'	8–27
16SUNI-R	5' GTGTGACGGGCGGTGTGTAC 3'	1410–1391
UNI16S-R	5' AAGGAGGTGATCCAGCCGCA 3'	1540–1521 ^b

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

^b 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.

* Corresponding author. Mailing address: Institute of Veterinary Bacteriology, Laenggass-Str. 122, CH-3012 Bern, Switzerland. Phone: 41-31-6312369. Fax: 41-31-6312634. Electronic mail address: Kuhnert@vbi.unibe.ch.

This work was supported by the Foundation Research 3R (grant 37/92) and by the Priority Program Biotechnology of the Swiss National Science Foundation (grant 5002-038920).

REFERENCES

1. Blaha, T. 1989. Applied veterinary epidemiology. Gustav Fischer Press, New York.
2. Carbon, P., C. Ehresmann, B. Ehresmann, and J. P. Ebel. 1979. The complete nucleotide sequence of the ribosomal 16-S RNA from *Escherichia coli*. Eur. J. Biochem. **100**:399–410.
3. Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. E. Farrow. 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. **44**:812–826.
4. Hatheway, C. L. 1990. Toxigenic clostridia. Clin. Microbiol. Rev. **3**:66–98.
5. Hippe, H., J. R. Andreesen, and G. Gottschalk. 1991. The Genus *Clostridium*—nonmedical, p. 1800–1866. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York.
6. Hutson, R. A., D. E. Thomson, and M. D. Collins. 1993. Genetic interrelationships of saccharolytic *Clostridium botulinum* types B, E and F and related clostridia as revealed by small-subunit rRNA gene sequences. FEMS Microbiol. Lett. **108**:103–110.
7. Pitcher, D. G., N. A. Saunders, and R. J. Owen. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. **8**:151–156.
8. Smith, L. D. S. 1991. The genus *Clostridium*—Medical, p. 1867–1878. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York.