Clostridium novyi is divided into three types, A, B and C. Types A and B are pathogenic and type C is generally recognized as non-pathogenic to laboratory animals. C. novyi type A causes gas gangrene in man and animals and C. novyi type B frequently causes infectious necrotic hepatitis in sheep. Clostridium haemolyticum is the causal organism of barillary haemoglobinuria in cattle (Smith, 1975). Although the disease caused by C. haemolyticum is quite unlike those caused by C. novyi types A and B, these bacteria are very similar in their biological characteristics. In particular, it is difficult to distinguish between C. novyi type B and C. haemolyticum. Although C. haemolyticum produces more of the beta toxin and has a higher sensitivity to mitomycin C than C. novyi type B (Nakamura et al., 1975), its major lethal toxin is unlike the haemolytic lecithinase (beta toxin) of C. novyi type B.Oakley et al. (1947) questioned whether C. haemolyticum was a member of C. novyi type B or an independent type, because the main difference between these two species was the production of alpha (necrotizing) toxin, observed only in C. novyi types A and B. Moreover, it has been demonstrated that alpha toxigenesis in C. novyi type B and C. haemolyticum is intimately related to phage infection; a phage-cured C. novyi type B, which ceased to produce its alpha toxin, produced it again after reinfection with the phage (Eklund et al., 1976) and C. haemolyticum infected with the phage of C. novyi type A produced the alpha toxin (Schallehn & Eklund, 1980). Cato et al. (1982) also demonstrated that C. novyi type B and C. haemolyticum were indistinguishable in PAGE analysis of soluble cellular proteins; the PAGE patterns of C. novyi type B, C. haemolyticum and Clostridium botulinum types C and D were very similar. Moreover, Nakamura et al. (1983) reported that C. novyi type B and C. haemolyticum comprise one genetically homologous group by DNA–DNA in vitro molecular hybridization.

16S rRNA gene analysis is now well established as a standard method for the identification of species, genera and families of bacteria (Woese, 1987) and for the construction of phylogenetic trees. Recently, Stackebrandt et al. (1999) reported that C. haemolyticum and C. novyi occupied different positions in the 16S rRNA phylogenetic tree. According to these data, C. haemolyticum clustered with C. botulinum types C

...
containing 4 mg lysozyme ml−1 were resuspended in 150 µl PBS and cultured in CLB broth (Azechi et al., 1962) under anaerobic condition at 37 °C. Bacterial cells in late exponential phase (1 ml culture) were centrifuged and the supernatant was removed. Cell pellets were washed twice with PBS and re-suspended in 1 ml of PBS. The bacterial cells were then resuspended in 0.85% NaCl in order to lyse bacteria. The suspensions were then mixed with 1 ml of buffer (0.85% NaCl, 2 mM sodium azide) and incubated at 37 °C for 1 h. The mixtures were then centrifuged at 10,000 g for 10 min and the supernatants were removed.

**Table 1. Primers used for amplification and/or sequencing**

All of the primers, except for 16SUNI-L and UNI16S-R reported by Kuhnert et al. (1996), were designed from the *C. novyi* type A 16S rDNA sequence.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SUNI-L</td>
<td>5'-AGAGTTTGTATCGTGTCAG-3'</td>
<td>8–27</td>
</tr>
<tr>
<td>UNI16S-R</td>
<td>5'-AAGAGGTGACACGCGGCA-3'</td>
<td>1540–1521</td>
</tr>
<tr>
<td>16SRNA1</td>
<td>5'-AAGCCTTCATCCTACCTC-3'</td>
<td>306–321</td>
</tr>
<tr>
<td>16SRNA2</td>
<td>5'-CGGATCGTAAATCTGTGTC-3'</td>
<td>322–349</td>
</tr>
<tr>
<td>16SRNA3</td>
<td>5'-TACCCCCCGACACCTAGT-3'</td>
<td>743–725</td>
</tr>
<tr>
<td>16SRNA4</td>
<td>5'-AAGCAATTAAGTGATCCGCGC-3'</td>
<td>764–772</td>
</tr>
<tr>
<td>16SRNA5</td>
<td>5'-GCTCGTTGTACAGCCATGT-3'</td>
<td>1156–1136</td>
</tr>
<tr>
<td>16SRNA6</td>
<td>5'-CAGTTCCGATTGATGGCTG-3'</td>
<td>1198–1216</td>
</tr>
</tbody>
</table>

* Numbers indicate positions relative to the *E. coli* 16S rDNA sequence (GenBank accession no. J01859).

and D, whereas one *C. novyi* strain group with *Clostridium sporogenes*. Therefore, it is very interesting to note where *C. novyi* type B and C are located in the 16S rDNA phylogenetic tree. To clarify the phylogenetic positions of *C. novyi* types A, B and C and *C. haemolyticum*, the sequences of a 1465 bp region encoding 16S rDNA of seven *C. novyi* strains and two *C. haemolyticum* strains were determined.

*C. haemolyticum* strains ATCC 9650T and ATCC 9652, *C. novyi* type A strains JCM 1406T, ATCC 19402 and 140 (provided by R. Azuma, Tokyo University of Agriculture, Tokyo, Japan), *C. novyi* type B strains ATCC 25758, CN 1025 (Japanese vaccine strain) and IRP 307 and *C. novyi* type C strain ATCC 27323 were cultured in CLB broth (Azechi et al., 1962) under anaerobic condition at 37 °C. Bacterial cells in late exponential phase (1 ml culture) were centrifuged and resuspended in 150 µl sucrose/TE (25%, w/v, sucrose, 10 mM Tris/HCl, 1 mM EDTA, 150 mM NaCl) containing 4 mg lysozyme ml−1. DNA extraction and purification were carried out according to Takeuchi et al. (1997). For PCR, primers 16SUNI-L and UNI16S-R (Kuhnert et al., 1996) were used to amplify a 1500 bp region of the 16S rDNA sequences. Reaction mixtures (100 µl), consisting of 1× PCR buffer (MgCl2-free), 2.5 mM MgCl2, 0.2 mM dNTP mixture, 2.5 U LA *Taq* DNA polymerase (LA *Taq* kit; TaKaRa), 10 pmol each primer and 1 µl template DNA solution, were subjected to 30 cycles of amplification in a PCR thermal cycler (PC-700; Asteck). The amplification cycle was as follows: preheating at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 1.5 min. The final extension was performed at 72 °C for 7 min. The amplified products were purified after electrophoresis in 20% (w/v) agarose gel. The purified PCR product was then sequenced directly using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) with approximately 400 ng template DNA and 4 pmol primer per reaction (Table 1). Automated DNA sequencing of sample DNA was performed on a DNA sequencer (model 373A; PE Biosystems). All sequences were confirmed by sequencing both strands.

The 16S rDNA sequences of *C. novyi* type B strains ATCC 25758, CN 1025 and IRP 307 were identical and the sequences of *C. haemolyticum* strains ATCC 9650T and ATCC 9652 were also identical. Those of *C. novyi* type A strains JCM 1406T, ATCC 19402 and 140 were also identical. Moreover, the sequences of *C. novyi* type B strains were completely identical to those of *C. haemolyticum* strains ATCC 9650T and ATCC 9652; they differed by 1 bp (level of similarity > 99.9%) from *C. novyi* type C strain ATCC 27323 and they were 98.7% homologous with *C. novyi* type A strain JCM1406T (relative positions 28–1520 of the *Escherichia coli* 16S rDNA sequence). Although Stackebrandt et al. (1999) reported that one strain of *C. novyi* grouped with *C. sporogenes*, our study showed that the type strain of *C. novyi* had higher similarity to *C. botulinum* types D (98.8%) and C (98.6%) than to *C. sporogenes* (90.9%) (relative positions 28–1497 of the *E. coli* 16S rDNA sequence).

In order to analyse the phylogenetics of *C. novyi* types A, B and C and *C. haemolyticum*, their 16S rDNA sequences were compared with GenBank data for other clostridia. The region from positions 28 to 1410 (*E. coli* numbering) was used for the calculation. As it was reported that *C. novyi* and *C. haemolyticum* belonged to *Clostridium* cluster I (Collins et al., 1994; Stackebrandt et al., 1999), sequence data for *Clostridium chauvoei*, *Clostridium septicum*, *Clostridium carnis*, *Clostridium perfringens*, *Clostridium tetani*, *C. sporogenes*, *C. botulinum* type A and B (non-protoelectic) and C, D, E and F (non-protoelectic) strains were used as representatives of *Clostridium* cluster I. *Clostridium sordellii*, in cluster XI, was used as an outgroup of cluster I. Phylogenetic analysis was performed with the software CLUSTAL W (Thompson et al., 1994). The evolutionary distance matrix of the aligned sequences was calculated using the two-
Paraclonal analysis was performed on the 16S rDNA sequences of various strains of Clostridium spp. to determine their phylogenetic relationships. The 16S rDNA sequences of the organisms were aligned, and the reliability of the tree topology was estimated using the neighbour-joining method (Saitou & Nei, 1987) and the bootstrap method (Felsenstein, 1985). A tree was constructed from the distance matrix parameter model for multiple substitutions (Kimura, 1980). Bootstrap values (expressed as percentages of 1000 replications) were calculated to assess the stability of the branching relationships.

Fig. 1. Phylogenetic relationships of C. novyi types A, B and C and C. haemolyticum to other clostridia based on 16S rDNA sequences. The 16S rDNA sequences were trimmed, which resulted in fragments ranging from positions 28 to 1410 (E. coli numbering). The outgroup is C. sordellii. GenBank accession numbers of the 16S rDNA sequences of the organisms are given in parentheses. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points. Bar, 0·1 (evolutionary distance).

References


