NOTE

Phylogenetic positions of *Clostridium novyi* and *Clostridium haemolyticum* based on 16S rDNA sequences

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The partial sequences (1465 bp) of the 16S rDNA of *Clostridium novyi* types A, B and C and *Clostridium haemolyticum* were determined. *C. novyi* types A, B and C and *C. haemolyticum* clustered with *Clostridium botulinum* types C and D. Moreover, the 16S rDNA sequences of *C. novyi* type B strains and *C. haemolyticum* strains were completely identical; they differed by 1 bp (level of similarity > 99.9%) from that of *C. novyi* type C, they were 98.7% homologous to that of *C. novyi* type A (relative positions 28–1520 of the *Escherichia coli* 16S rDNA sequence) and they exhibited a higher similarity to the 16S rDNA sequence of *C. botulinum* types D and C than to that of *C. novyi* type A. These results suggest that *C. novyi* types B and C and *C. haemolyticum* may be one independent species generated from the same phylogenetic origin.

Keywords: *Clostridium novyi* types A, B and C, *Clostridium haemolyticum*, 16S rRNA analysis

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 bacillary 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 identical to 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 pro-

duction 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 re-infection 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 rDNA phylogenetic tree. According to these data, *C. haemolyticum* clustered with *C. botulinum* types C

The DDBJ accession numbers for the 16S rDNA sequences of *C. haemolyticum* strain ATCC 9650^T, *C. novyi* type B strain ATCC 25758, *C. novyi* type A strains JCM 1406^T and ATCC 19402 and *C. novyi* type C strain ATCC 27323 are AB037910, AB035087, AB045606, AB041866 and AB041865, respectively.

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.

Primer	Sequence	Position*
16SUNI-L	5'-AGAGTTTGATCATGGCTCAG-3'	8-27
UNI16S-R	5'-AAGGAGGTGATCCAGCCGCA-3'	1540-1521
16SRNA1	5'-AAAACCTTCATCACTC-3'	306-321
16SRNA2	5'-CGGATCGTAAAACTCTGTC-3'	322-349
16SRNA3	5'-TACCCCCCGACACCTAGT-3'	743-725
16SRNA4	5'-AACGCATTAAGTATTCCGC-3'	764-772
16SRNA5	5'-GCTCGTTGTACCAGCCATTGT-3'	1156-1136
16SRNA6	5'-CAGTTCGATTGTAGGCTG-3'	1198-1216

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

and D, whereas one *C. novyi* strain grouped with *Clostridium sporogenes*. Therefore, it is very interesting to note where *C. novyi* types 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 9650^T and ATCC 9652, *C. novyi* type A strains JCM 1406^T, 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/TES (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 (Mg²⁺-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 2.0% (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 9650^T and ATCC 9652 were also identical. Those of *C. novyi* type A strains JCM 1406^T, 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 9650^T 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 JCM1406^T (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-proteolytic) and C, D, E and F (non-proteolytic) 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-

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).

parameter model for multiple substitutions (Kimura, 1980). A tree was constructed from the distance matrix by the neighbour-joining method (Saitou & Nei, 1987). The reliability of the tree topology was estimated by the bootstrap method (Felsenstein, 1985). Although Stackebrandt *et al.* (1999) reported that one strain of *C. novyi* clustered with *C. sporogenes*, in this study, *C. novyi* types A, B and C and *C. haemolyticum* clustered with *C. botulinum* types C and D. In particular, *C. novyi* types B and C and *C. haemolyticum* comprised one genetically homologous group (Fig. 1). In addition, they exhibited a higher similarity to *C. botulinum* types D (99.4%) and C (99.1%) than to *C. novyi* type A (98.5%). Nakamura *et al.* (1983) reported that four strains of *C. novyi* type B had 89–95% DNA–DNA homology to *C. haemolyticum*, 28–37% to *C. novyi* type A and 68–70% to *C. botulinum* type C. Our results agree with their report that *C. novyi* type B and *C. haemolyticum* comprise one genetically homologous group. Moreover, the DNA G + C contents of *C. novyi* type B and *C. haemolyticum* did not differ (26 mol%), but *C. novyi* type C and *C. novyi* type A had DNA G + C contents of 27 and 29 mol%, respectively

(Johnson & Francis, 1975). It has been suggested that *C. haemolyticum* should be placed in the *C. novyi* species and be referred to as *C. novyi* type D (Oakley & Warrack, 1959). However, *C. novyi* types B and C and *C. haemolyticum* may be one independent species, generated from the same phylogenetic origin, since the disease caused by each bacterium could be determined by infection with toxin-related bacteriophages, the 16S rDNA sequences of *C. novyi* type B and *C. haemolyticum* were identical and these sequences exhibited a higher similarity to those of *C. botulinum* types D and C than to those of *C. novyi* type A.

As *C. novyi* types A and B and *C. haemolyticum* are pathogenic and have very similar biological characteristics, the identification of these bacteria is a difficult and time-consuming task. In this respect, specific PCR systems for *C. novyi* type B and *C. haemolyticum* and for *C. novyi* type A based on the 16S rDNA sequences presented in this paper and a specific PCR system for *C. novyi* types A and B based on the alpha toxin gene sequence of *C. novyi* reported by Hofmann *et al.* (1995) should be developed for the identification of these bacteria. Results of these three PCR systems will enable rapid and simple identification of these bacteria. Moreover, isolation of the bacteria is very difficult since they require strict anaerobic conditions and clinical specimens are often contaminated with other anaerobic bacteria, including clostridia from soil, which grow faster than these pathogenic bacteria in culture medium. Therefore, direct detection systems of the pathogenic bacteria without cultivation are desirable. In this way, our work based on molecular genetics will help improve diagnostic tools for these bacteria.

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