Clostridium sardiniense Prévot 1938 and *Clostridium absonum* Nakamura *et al.* 1973 are heterotypic synonyms: evidence from phylogenetic analyses of phospholipase C and 16S rRNA sequences, and DNA relatedness

Xingmin Wang,[†] Tsuneo Maegawa,[‡] Tadahiro Karasawa,[§] Eijiro Ozaki and Shinichi Nakamura

Correspondence

Tadahiro Karasawa
karasawa@mhs.mp.
kanazawa-u.ac.jp

Department of Bacteriology, Graduate School of Medical Science, Kanazawa University,
13-1 Takara-machi, Kanazawa 920-8640, Japan

Clostridium sardiniense Prévot 1938 and *Clostridium absonum* Nakamura *et al.* 1973 have long been considered similar in terms of their biological and biochemical properties, but their taxonomic positions have not been clarified by DNA–DNA hybridization studies or rigorous analysis of 16S rRNA genes. In the present study, DNA–DNA hybridization analysis revealed that *C. absonum* strains DSM 599^T, DSM 600 and KZ 1544 shared 83.0–86.3% DNA relatedness with *C. sardiniense* DSM 2632^T. 16S rRNA gene sequence analysis showed that the *C. absonum* strains also shared high identity with *C. sardiniense* DSM 2632^T (99.7, 99.3 and 99.8% for DSM 599^T, DSM 600 and KZ 1544, respectively), implying that *C. absonum* and *C. sardiniense* are synonyms. In addition, alignment of the inferred amino acid sequences for phospholipase C (PLC) indicated 96.5% identity between PLCs from *C. sardiniense* and *C. absonum*, but relatively low identity with other clostridial species. These results strongly suggest that the species *C. sardiniense* and *C. absonum* should be united, with the name *C. sardiniense* having priority.

Clostridia produce lecithinases known as phospholipase C (PLC) (Titball, 1999; Jepson & Titball, 2000). The best characterized clostridial PLC is produced by *Clostridium perfringens*. Because *C. perfringens* PLC is toxic to mammals, it is termed an alpha-toxin and considered a major virulence

factor. However, there are still many PLCs expressed by other clostridial species. Previous results suggest that these PLCs are similar but retain species-specific structure and function (Clark *et al.*, 2003; Karasawa *et al.*, 2003). *Clostridium absonum* was first described by Nakamura *et al.* (1973) and, unlike *C. perfringens*, is primarily isolated from soil and animal faeces. Later, this species was also identified as a causative agent of gas gangrene (Nakamura *et al.*, 1979; Masaki *et al.*, 1988). Although the morphological and biochemical properties of *C. absonum* are similar to those of *C. perfringens* (Nakamura *et al.*, 1973; Hayase *et al.*, 1974), *C. absonum* is easily differentiated from *C. perfringens* by the lecithinase (PLC) neutralization test. In this assay, PLC produced by *C. absonum* on half-antitoxin egg-yolk agar cannot be completely neutralized by *C. perfringens* type A antitoxin as is the *C. perfringens* PLC (Nakamura *et al.*, 1973). Recently, our research group isolated the PLC gene (*caa*) from *C. absonum* and characterized the crystal structure of the expressed protein (Caa) (Clark *et al.*, 2003). In the course of these studies, particular attention was paid to *Clostridium sardiniense* since this species is similar to *C. absonum* in biological and biochemical properties (Cato *et al.*, 1986). *C. sardiniense* was initially described by Prévot in 1938 as a PLC-producing clostridial species. It is

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[†]Present address: Department of Medicine, Infectious Diseases Section, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190, USA.

[‡]Present address: JCS Inc., 5-758 Nakamozu-cho, Sakai 591-8023, Japan.

[§]Present address: Department of Laboratory Sciences, School of Health Sciences, Kanazawa University, 5-11-80 Kodatsuno, Kanazawa 920-0942, Japan.

Abbreviation: PLC, phospholipase C.

The GenBank/EMBL/DDBJ accession numbers for the sequence of the gene encoding phospholipase C of *C. sardiniense* DSM 2632^T and for the 16S rRNA gene sequences of *C. sardiniense* DSM 2632^T and *C. absonum* (DSM 599^T, DSM 600 and KZ 1544) are AB162962, AB161367–AB161368 and AB161369–AB161374, respectively.

Results of DNA–DNA hybridizations are available as supplementary material in IJSEM Online.

frequently isolated from soil samples (Rodriguez *et al.*, 1993) and the faeces of infants (Borriello, 1980), although no association with gas gangrene has been reported. We isolated the PLC gene from *C. sardiniense* and found it to be highly homologous to *caa*, suggesting both genes may derive from the same species. Based on 16S rRNA gene sequence data, *C. sardiniense* has been classified relatively far from *C. absonum* in *Clostridium* 16S rRNA Cluster I (Collins *et al.*, 1994). However, no DNA–DNA hybridization studies have been performed to confirm this phylogenetic relationship. Therefore, to clarify the taxonomic relationship between three strains of *C. absonum* (DSM 599^T, DSM 600 and KZ 1544) and *C. sardiniense* DSM 2632^T, we compared inferred amino acids for the respective PLCs and 16S rRNA genes, and performed DNA–DNA hybridization analyses.

C. sardiniense DSM 2632^T (= ATCC 33455^T = VPI 2971^T), *C. absonum* DSM 599^T (= ATCC 27555^T = NCTC 10984^T = CIP 104302^T = JCM 1381^T) (Clark *et al.*, 2003), *C. absonum* DSM 600, *C. absonum* KZ 1544 (Nakamura *et al.*, 1979), *C. perfringens* KZ 221 (Tsutsui *et al.*, 1995; Karasawa *et al.*, 2003), *Clostridium bifermentans* KZ 1012 (Karasawa *et al.*, 2003) and *Clostridium sordellii* NCIMB 10717^T (= ATCC 9714^T) (Karasawa *et al.*, 2003) were used in this study. For extraction of whole-cell DNA, bacterial strains were cultured in 40 ml of GAM broth (Nissui Pharmaceutical) at 37 °C for 16 h. Extraction and purification of whole-cell DNA was performed as described previously (Wang *et al.*, 2000; Karasawa *et al.*, 2003). As a preliminary experiment, a 900 bp fragment of the *plc* gene of *C. sardiniense* was amplified by using the KAG209 (5'-TGGGATGGAAAAGATTGATGGAACAGG-3') and KAG210 (5'-TTTCTCTTTTCTTATCCACATATTCTTG-TATATC-3') primer set for highly conserved regions of clostridial *plc* genes (Karasawa *et al.*, 2003). The sequence of the 900 bp fragment showed high identity with the *C. absonum caa* gene (Clark *et al.*, 2003). Therefore, the whole open reading frame was amplified by using the KAG188 (5'-CCAACCACAATTATCTTCTTTACTACCT-3') and KAG201 (5'-CACATCCTGTTATCTCTTCCTATCA-3') primer set that encodes sequences flanking *caa*. The PCR mixture consisted of 0.2 mM (each) deoxynucleotide triphosphates, 50 pmol of each primer, 100 ng of whole-cell DNA and 2.5 units of TaKaRa Ex *Taq* (Takara Shuzo) in a 50 µl of buffer. The PCR was performed as follows: pre-denaturation at 94 °C for 1 min, followed by 30 cycles at 94 °C for 20 s, 58 °C for 15 s and 72 °C for 45 s on a GeneAmp PCR System 9700 (Applied Biosystems). PCR products were electrophoresed on a 1 % agarose gel and a fragment of approximately 1300 bp was extracted by using a QIAquick Gel Extraction kit (Qiagen). The purified fragment was ligated to a pCRII-TOPO TA cloning vector (Invitrogen) and transformed into competent *Escherichia coli* TOP 10F' cells according to the manufacturer's instructions. Transformants were screened on a 2 × YT plate containing 50 µg ampicillin ml⁻¹ and 5 % egg yolk. Several colonies were identified that showed the characteristic lecithinase reaction of opacity around a colony. The plasmid

of a PLC-positive transformant was extracted with a QIAprep Spin Miniprep kit (Qiagen), sequenced in both directions using a BigDyeTM Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) with synthetic primers and electrophoresed on an ABI Prism 310 Genetic Analyser (Applied Biosystems). A 1519 bp fragment of the 16S rRNA gene was amplified by using KAG268 (5'-TTTAAATTGAGAGTTTGATCCTGGCTCA-3') and KAG269 (5'-AGAAAGGAGGTGATCCAGCCGCA-3') as a primer set, designed from conserved sequences identified by comparing several clostridial 16S rRNA genes. The amplification, cloning and sequencing of the 16S rRNA genes were the same as described for the *C. sardiniense plc* gene except PCR was carried out using a two-step procedure of 30 cycles at 94 °C for 20 s and 72 °C for 40 s, after pre-heating at 94 °C for 1 min. To prepare DNA probes for DNA–DNA hybridization analysis, chromosomal DNA was labelled by random priming using digoxigenin–dUTP according to the manufacturer's instructions for the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Molecular Biochemicals). DNA–DNA hybridization was carried out as described by Ezaki *et al.* (1989) with some modifications. Purified chromosomal DNA was diluted to 100 µg ml⁻¹ with H₂O and denatured by boiling for 10 min at 100 °C. Denatured DNA was diluted to 20 µg ml⁻¹ with PBS (8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2, 137 mM NaCl, 2.7 mM KCl) containing 0.1 M MgCl₂, and aliquots (100 µl) were immobilized onto NUNC-immuno microplates (Nalge Nunc International) by incubation at 37 °C for 3 h. Duplicate controls were made for each DNA sample. After aspirating the coating solution, 200 µl of pre-hybridization solution [2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 5 × Denhardt's solution, 25 % formamide, 200 µg salmon sperm DNA ml⁻¹] was added to wells and incubated at 37 °C for 3 h. Before hybridization with labelled DNA probe, wells were washed three times with 2 × SSC. One-hundred microlitres of hybridization solution (2 × SSC, 5 × Denhardt's solution, 3 % dextran sulfate, 25 % formamide, 50 µg denatured salmon sperm DNA ml⁻¹, 0.5 µg denatured digoxigenin–dUTP labelled probe ml⁻¹) was added to wells and incubated at 37 °C overnight. The wells were washed three times with 300 µl of 2 × SSC and once with 300 µl of washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3 % Tween 20). Detection of dsDNA was done by using an anti-digoxigenin–alkaline phosphatase conjugate included in the kit. Finally, 100 µl of *p*-nitrophenyl phosphate disodium (mg ml⁻¹) (in 0.05 M Tris/HCl, pH 10.0, 0.1 M NaCl, 0.5 mM MgCl₂) was dispensed into the wells, with a 30 min incubation at 37 °C, and the reaction was stopped by adding 100 µl of 3 M NaOH. Absorbance was measured with a microplate reader EAR 340 AT (Tecan) at 405 nm with a reference wavelength of 620 nm. The assay was repeated at least three times for all strains with results shown as the mean ± SD. Multiple alignments of sequences were created by using the CLUSTAL W method and phylogenetic trees were calculated by using the neighbour-joining method

Fig. 1. Unrooted phylogenetic tree based on PLC amino acid sequences. Bar, 0.1 substitution per site.

contained within an online software package (<http://www.ddbj.nig.ac.jp/search/clustalw-e.html>). Unrooted phylogenetic trees were constructed with TreeView (version 1.6.6 for Windows) software.

The *plc* gene of *C. sardiniense* DSM 2632^T consisted of 1197 nt encoding 398 aa residues. The inferred protein had a molecular mass of 45.7 kDa and a pI of 5.26. Between the *plc* gene of *C. sardiniense* and the *caa* gene of *C. absonum* DSM 599^T (Clark *et al.*, 2003), nucleotide differences were found at 57 positions, resulting in coding changes for 14 aa. Alignment of amino acid sequences revealed that the *C. sardiniense* PLC had 96.5% identity with Caa and showed relatively low identity with PLCs from *C. perfringens* (Cpa), *C. bifermentans* (Cbp) and *C. sordellii* (Csp) (Karasawa *et al.*, 2003). A phylogenetic tree based on the amino acid sequences of PLCs from related clostridial species is shown in Fig. 1. The PLC of *C. sardiniense* was closely related to that of *C. absonum*. Not surprisingly, since *C. bifermentans* and *C. sordellii* are classified in the same *Clostridium* 16S rRNA cluster XI by Collins *et al.* (1994), Cbp appeared closely related to Csp.

Cbp and Csp, however, were only loosely related to Cpa, Caa and the *C. sardiniense* PLC.

As mentioned above, Caa could not be completely neutralized with antiserum to Cpa (Nakamura *et al.*, 1973). When the half-antitoxin egg-yolk agar test (Willis & Hobbs, 1958) was used to compare PLCs of *C. sardiniense* and *C. absonum*, the *C. sardiniense* DSM 2632^T PLC was also incompletely neutralized by antiserum to *C. perfringens* PLC (data not shown).

The 16S rRNA genes from *C. sardiniense* DSM 2632^T and *C. absonum* DSM 599^T, DSM 600 and KZ 1544 were sequenced. Alignment of the 16S rRNA gene sequences revealed that the *C. absonum* strains shared extremely high identity with the sequence of *C. sardiniense* DSM 2632^T (99.3–99.9%). A phylogenetic tree based on 16S rRNA gene sequences is shown in Fig. 2. *C. absonum* strains DSM 599^T, DSM 600 and KZ 1544 appeared closely related to *C. sardiniense* DSM 2632^T. According to Collins *et al.* (1994), although *C. sardiniense*, as well as *C. absonum*, is classified in *Clostridium* 16S rRNA cluster I, *C. sardiniense* is only loosely associated with *C. absonum*. Of note is that

Fig. 2. Unrooted phylogenetic tree based on 16S rRNA gene sequences. Bar, 0.1 substitution per site. The microheterogeneity of the 16S rRNA gene from *C. absonum* and *C. sardiniense* strains did not affect the phylogenetic tree.

the 16S rRNA gene sequences for both *C. absonum* and *C. sardiniense* in GenBank/EMBL/DDBJ (accession nos X77842 and X73446, respectively) contain several undetermined nucleotide residues, probably resulting in the loose association reported by Collins *et al.* (1994). Indeed, we also found a few undetermined nucleotide residues in preliminary sequences for these 16S rRNA genes when we used a direct method with PCR products. Therefore, the PCR products of the 16S rRNA genes were cloned into a plasmid vector and three to six clones were sequenced per strain. Our results indicated two kinds of 16S rRNA gene with microheterogeneity in every strain tested. The two different clones found in *C. sardiniense* DSM 2632^T were defined as DSM 2632^{C1} (four of six sequenced clones) and DSM 2632^{C2} (two of six sequenced clones). Differences found at six nucleotide positions between DSM 2632^{C1} and DSM 2632^{C2} were: G819, C880, C1001, T1003, G1010 and G1489 in DSM 2632^{C1} substituted by A819, T880, T1001, C1003, A1010 and A1489 in DSM 2632^{C2}. Microheterogeneities were also found in three strains of *C. absonum* with C1001 and G1010 in DSM 599^{C1} (two of three clones) substituted by T1001 and A1010 in DSM 599^{C2} (one of three clones); C225, C359, C442, A819, C821 and C982 in DSM 600^{C1} (two of three clones) substituted by T225, T359, T442, G819, T821 and T982 in DSM 600^{C2} (two of three clones); and T1001, T1003 and G1010 in KZ 1544^{C1} (two of three clones) substituted by C1001, C1003 and A1010 in KZ 1544^{C2} (one of three clones). Microheterogeneities have been reported in the 16S *rrn* operon of *E. coli* (Carbon *et al.*, 1979), the 23S *rrn* operon of *Clostridium botulinum* type A (East *et al.*, 1992) and the 5S *rrn* operon of *Bacillus* species (Raue *et al.*, 1977). Although microheterogeneity does not pose a problem for phylogenetically distant organisms, such considerations become increasingly important when close genealogical relationships exist (East *et al.*, 1992). However, despite the microheterogeneity, the phylogenetic analysis of 16S rRNA gene sequences implies that *C. absonum* and *C. sardiniense* can be classified at equal taxonomic positions.

High levels of DNA relatedness were detected between *C. sardiniense* DSM 2632^T and the three strains of *C. absonum*: (83.0, 84.7 and 86.3 % for DSM 599^T, DSM 600 and KZ 1544, respectively) (results of DNA–DNA hybridizations are available as supplementary material in IJSEM Online). However, relatively low levels of DNA relatedness (18.2–28.0 %) were detected between *C. perfringens* KZ 221 and all strains of *C. absonum* and *C. sardiniense*. A relatively high level of DNA relatedness (64.6 %) was found for *C. bifermentans* and *C. sordellii*, which are considered closely related based on data from 16S rRNA gene sequences (Collins *et al.*, 1994) and DNA–DNA hybridization analyses (Nakamura *et al.*, 1975). However, *C. bifermentans* and *C. sordellii* showed low level DNA relatedness to *C. absonum*, *C. sardiniense* and *C. perfringens*. DNA–DNA hybridization also indicated that *C. absonum* is equal to *C. sardiniense* in its phylogenetic position.

Interestingly, phylogenetic trees exhibited excellent correlation whether based on the inferred amino acid sequences of PLCs, 16S rRNA genes or degree of DNA relatedness in hybridization experiments. Our results suggest that the evolution of PLC closely parallels that of 16S rRNA genes in PLC-producing clostridia. Genes encoding PLC could be as useful for phylogenetic analysis of PLC-producing clostridia as 16S rRNA genes. Similarly, the phylogenetic tree based on 16S rRNA gene sequences of PLC-producing clostridia should provide good clues for analysing the primary structure and function of clostridial PLCs.

C. sardiniense and *C. absonum* were first described by Prévot (1938) and Nakamura *et al.* (1973), respectively. Cato *et al.* (1986) recognized that *C. sardiniense* was similar to *C. absonum* in its biological and biochemical properties, and further noted that '*C. sardiniense* is differentiated from *C. absonum* by motility but since some strains labeled *C. absonum* have been found to be motile, clear separation of the species must await DNA homology studies'. However, until this study, the taxonomic position of both species had not been clarified. DNA hybridization studies had not been conducted, and the 16S rRNA gene sequences for *C. absonum* DSM 599^T and *C. sardiniense* DSM 2632^T in GenBank/EMBL/DDBJ (accession nos X77842 and X73446, respectively) contained undetermined nucleotides, resulting in a reportedly loose association between these species (Collins *et al.*, 1994). The present study has demonstrated that *C. absonum* strains DSM 599^T, DSM 600 and KZ 1544 share 83.0–86.3 % DNA relatedness with *C. sardiniense* strain DSM 2632^T. Moreover, 16S rRNA gene sequence analysis showed that all *C. absonum* strains in this study shared high identity with *C. sardiniense* DSM 2632^T (99.7, 99.3 and 99.8 % for DSM 599^T, DSM 600 and KZ 1544, respectively), implying that *C. absonum* and *C. sardiniense* can now be classified at equal taxonomic positions. In addition, alignment of inferred amino acids for PLCs indicated 96.5 % identity between the *C. sardiniense* and *C. absonum* proteins, but relatively low identity to other clostridial species. These results strongly suggest that the names *C. sardiniense* and *C. absonum* are synonyms. Therefore, according to Rule 24b of the International Code of Nomenclature of Bacteria, we propose that *C. absonum* be recognized as a later synonym of *C. sardiniense*, and that the species *C. sardiniense* and *C. absonum* should be united, with the name *C. sardiniense* having priority.

Emended description of *Clostridium sardiniense* Prévot 1938

Later heterotypic synonym: *Clostridium absonum* Nakamura *et al.* 1973 (Approved Lists 1980).

The description of the species is as given by Cato *et al.* (1986), based mainly on the studies of Nakamura *et al.* (1973) and Holdeman *et al.* (1977).

The type strain is DSM 2632^T (=ATCC 33455^T = VPI 2971^T).

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