The GenBank/EMBL/DDBJ accession numbers for the sequence of the gene encoding phospholipase C of \textit{C. sardiniense} DSM 2632\textsuperscript{T} and for the 16S rRNA gene sequences of \textit{C. sardiniense} DSM 2632\textsuperscript{T} and \textit{C. absonum} (DSM 599\textsuperscript{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.

Abbreviation: PLC, phospholipase C.

The results indicate that the species \textit{C. sardiniense} and \textit{C. absonum} are heterotypic synonyms: evidence from phylogenetic analyses of phospholipase C and 16S rRNA sequences, and DNA relatedness

\begin{itemize}
  \item \textit{Clostridium sardiniense} Prévot 1938 and \textit{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 \textit{C. absonum} strains DSM 599\textsuperscript{T}, DSM 600 and KZ 1544 shared 83.0–86.3% DNA relatedness with \textit{C. sardiniense} DSM 2632\textsuperscript{T}. 16S rRNA gene sequence analysis showed that the \textit{C. absonum} strains also shared high identity with \textit{C. sardiniense} DSM 2632\textsuperscript{T} (99.7, 99.3 and 99.8% for DSM 599\textsuperscript{T}, DSM 600 and KZ 1544, respectively), implying that \textit{C. absonum} and \textit{C. sardiniense} are synonyms. In addition, alignment of the inferred amino acid sequences for phospholipase C (PLC) indicated 96.5% identity between PLCs from \textit{C. sardiniense} and \textit{C. absonum}, but relatively low identity with other clostridial species. These results strongly suggest that the species \textit{C. sardiniense} and \textit{C. absonum} should be united, with the name \textit{C. sardiniense} having priority.

\item Clostridia produce lecinthinases known as phospholipase C (PLC) (Titball, 1999; Jepson & Titball, 2000). The best characterized clostridial PLC is produced by \textit{Clostridium perfringens}. Because \textit{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). \textit{Clostridium sardiniense} was first described by Nakamura et al. (1973) and, unlike \textit{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 \textit{C. absonum} are similar to those of \textit{C. perfringens} (Nakamura et al., 1973; Hayase et al., 1974), \textit{C. absonum} is easily differentiated from \textit{C. perfringens} by the lecinthinase (PLC) neutralization test. In this assay, PLC produced by \textit{C. absonum} on half-antitoxin egg-yolk agar cannot be completely neutralized by \textit{C. perfringens} type A antitoxin as is the \textit{C. perfringens} PLC (Nakamura et al., 1973). Recently, our research group isolated the PLC gene (ca) from \textit{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 \textit{Clostridium sardiniense} since this species is similar to \textit{C. absonum} in biological and biochemical properties (Cato et al., 1986). \textit{C. sardiniense} was initially described by Prévot in 1938 as a PLC-producing clostridial species. It is
\end{itemize}
frequently isolated from soil samples (Rodriguez et al., 1993) and the faces 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 599T, DSM 600 and KZ 1544) and *C. sardiniense* DSM 2632T, we compared inferred amino acids for the respective PLCs and 16S rRNA genes, and performed DNA–DNA hybridization analyses.

*C. sardiniense* DSM 2632T (= ATCC 33455T = VPI 2971T), *C. absonum* DSM 599T (= ATCC 27555T = NCTC 10984T = CIP 104302T = JCM 1381T) (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 bifermens* KZ 1012 (Karasawa et al., 2003) and *Clostridium ordellii* NCIMB 10717T (= ATCC 9714T) (Karasawa et al., 2003) were used in this study. For extraction of whole-cell DNA, bacterial colonies were identified that showed the characteristic colonies were washed three times with 300 μl of washing buffer (0.1 M NaCl, 0.25 M Tris/HCl, pH 7.3, containing 5 mg/ml denatured salmon sperm DNA ml−1) and once with 300 μl of pre-hybridization solution (15 M NaCl, 0.15 M sodium citrate). The denatured DNA 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 salmon sperm DNA ml−1 was added to wells and incubated at 37 °C for 3 h.
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. sardiniense* strains shared extremely high identity with the sequence of *C. absonum* 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 *Clostridium absonum* and *C. sardiniense* are synonyms.

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

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/DBJ (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⁺ were defined as DSM 2632C¹ (four of six sequenced clones) and DSM 2632C² (two of six sequenced clones). Differences found at six nucleotide positions between DSM 2632C¹ and DSM 2632C² were: G819, C880, C1001, T1003, G1010 and G1489 in DSM 2632C¹ substituted by A819, T880, T1001, C1003, A1010 and A1489 in DSM 2632C². Microheterogeneities were also found in three strains of *C. absonum* with C1001 and G1010 in DSM 599⁴ (two of three clones) substituted by T1001 and A1010 in DSM 599⁵ (one of three clones); C225, C359, C442, A819, C821 and C982 in DSM 600⁴ (two of three clones) substituted by T225, T359, T442, G819, T821 and T982 in DSM 600⁵ (two of three clones); and T1001, T1003 and G1010 in KZ 1544⁴ (two of three clones) substituted by C1001, C1003 and A1010 in KZ 1544⁵ (one of three clones). Microheterogeneities have been reported in the 16S rRNA operon of *E. coli* (Carbon *et al.*, 1979), the 23S rRNA operon of *Clostridium botulinum* type A (East *et al.*, 1992) and the 5S rRNA 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⁺ and the three strains of *C. absonum*: (83-0, 84-7 and 86-3 %) for DSM 599⁷, 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 RNA 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 Prévôt (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 599⁷ and *C. sardiniense* DSM 2632⁹ in GenBank/EMBL/DBJ (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 599⁷, DSM 600 and KZ 1544 share 83-0-86-3 % DNA relatedness with *C. sardiniense* strain DSM 2632⁹. Moreover, 16S rRNA gene sequence analysis showed that all *C. absonum* strains in this study shared high identity with *C. sardiniense* DSM 2632⁹ (99-7, 99-3 and 99-8 % for DSM 599⁷, 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 Prévôt 1938**


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 2632⁹ (= ATCC 33455⁹ = VPI 2971⁹).
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