Towards a phylogeny of the clostridia based on 16S rRNA sequences

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(Received 6 July 1993; revision received and accepted 22 July 1993)

Abstract: The 16S rRNA sequences of 17 species of the genus Clostridium were determined by direct sequencing of their PCR amplified genes. The sequences were aligned with those from other known clostridial species and representative low G+C Gram-positive relatives, and a phylogenetic tree was constructed. It was evident from the comparative sequence analysis that the genus Clostridium as presently constituted is phylogenetically extremely heterogeneous. This study corroborates and extends earlier findings in showing that many non-sporeforming bacteria are phylogenetically closely intermixed with Clostridium species. The taxonomic implications of the phylogenetic findings are discussed.

Key words: Clostridium; 16S rRNA; Phylogeny; Molecular systematics

Introduction

The genus Clostridium encompasses a collection of Gram-positive, obligately anaerobic, non-sulphate-reducing, sporeforming rod-shaped organisms [1]. Over 100 species are currently recognised [2] which display a wide range of phenotypes including for example psychrophiles, thermophiles and acidophiles, organisms which synthesise cytochromes and quinones, and others which are aerotolerant. In addition, the G+C content of chromosomal DNA ranges from approximately 21 to 54% [2] which is clearly too great to be encompassed by a single genus.

The earliest and most extensive phylogenetic study of the genus Clostridium was by Johnson and Francis [3] which demonstrated considerable diversity within the genus using DNA–rRNA pairing. Genealogical heterogeneity amongst the clostridia was subsequently confirmed by 16S-rRNA oligonucleotide cataloguing studies [4–7] which also indicated some clostridial species were phylogenetically intermixed with non-sporeforming taxa (e.g. eubacteria, peptococci, peptostreptococci). Direct sequencing of rRNA or rRNA genes has eclipsed oligonucleotide cataloguing in the study of bacterial phylogeny. It is now established that complete (or near complete) rRNA sequences provide far greater precision in resolving branching points in phylogenetic trees [7]. This is particularly important with the clostridia
which not only display marked heterogeneity but numerous relatively deep lineages. In recent years there has been a considerable accumulation in the number of full 16S rRNA sequences of the clostridia (e.g. [8-14]; see also Ribosomal Database Project [15]). As part of a systematic phylogenetic evaluation of the genus Clostridium, we have extended the available 16S rRNA data set by sequencing 17 clostridial species. In this article the results of a comparative analysis of these new sequences and those of other clostridial species and some representative non-sporeforming relatives are presented.

Materials and Methods

Determination of 16S rRNA gene sequences

The 16S rRNA gene sequences of seventeen Clostridium species were determined. Details of strains and their sources are given in Table 1. Chromosomal DNA was extracted and purified using the method described by Lawson et al. [16]. 16S rRNA gene sequences were determined directly from chromosomal DNA by amplifying the gene as two overlapping fragments of approximately 1 kb. The primers used for amplification and the PCR reaction conditions were as previously described [9]. DNA amplification products were purified using the Magic DNA Clean-up System (Promega) according to the manufacturer's instructions and sequenced directly using [α-35S]dATP and a Sequenasc Version 2.0 kit (United States Biochemicals) as previously described [9]. Sequencing products were separated on 55-cm wedge-shaped (0.2–0.6 mm) 6% acrylamide 7 M urea gels at 55°C using an LKB Macrophor 2010 sequencing unit operated at 50 W per gel.

Analysis of sequence data

Generated sequences were aligned and similarity values determined using the Phylip (version 3.5c) computer program run on Digital VAX-VMS (version V5.5). Nucleotide substitution rates (K_nuc) were calculated and an unrooted phylogenetic tree constructed using the Neighbour-joining method [17].

Nucleotide sequence accession numbers

The 16S rRNA gene sequences determined in the present study are available from EMBL Data Library under accession numbers shown in Table 1.

Results and Discussion

The 16S rRNA gene fragments of 17 clostridial species were amplified using PCR and their nucleotide sequences determined directly. The sequencing strategy employed resulted in the derivation of approximately 95% of their complete 16S rRNA primary structures (> 1450 nucleotides per gene). The derived 16S rRNA sequences have been deposited in EMBL Data Library (see Table 1). These new sequences were aligned with those of other published [4-14] and unpublished (Woese C.R., Yang, D., Mandelco, L., data from EMBL Library) clostridial sequences. The sequences of some representative
low G + C Gram-positive taxa known or considered to be related to clostridia were also included for comparative purposes (e.g. Atopobium, Eubacterium, Helio bacterium, Megasphaera, Peptostreptococcus, Peptococcus). Percent pairwise similarities for an approx. 1325-nucleotide region (ranging from positions 103 to 1428 of the Escherichia coli numbering system) were computed and used to construct a phylogenetic tree. Approximately 100 nucleotides at the 5' end of the rRNAs were omitted from these calculations to eliminate possible alignment errors due to the extremely variable V1 region. A dendrogram depicting the genealogical interrelationships of the strains using the Neighbour-joining method [17] is shown in Fig. 1.

The results of the treeing program confirm and extend earlier comparative rRNA studies in demonstrating marked genetic heterogeneity within the genus Clostridium. Of the 17 new strains sequenced, six fell into Clostridium rRNA homology group I of Johnson and Francis [3] which hosts the majority of the clostridial species, including the type species C. butyricum. Clostridium putrificum was found to be genealogically almost identical with the type strain of C. sporogenes (99.7% 16S rRNA sequence similarity based on 1512 nucleotides). This result is consistent with the reports of a close relationship between these species derived from DNA-rRNA pairing studies [3]. Clostridium paraputrificum, although genetically distinct, showed a specific affinity with C. aurantibutyricum, whereas C. puniceum displayed a relatively close relationship with C. beijerinckii and relatives (Fig. 1). Clostridium proteolyticum shared relatively high sequence relatedness with C. histolyticum and C. limosum (approx. 96% and 97% sequence similarity, respectively). These three species represent a distinct subline within rRNA group I clostridia. The remaining group I species (viz. C. cellulovorans, C. collagenovorans and C. sardiniensis) formed quite separate lines displaying no specific relationship with any of the other organisms examined.

Five clostridia species examined (viz. C. bifermentans, C. difficile, C. ghoni, C. irregularis and C. villosum) clustered with C. lituseburensense and relatives (equivalent to rRNA homology group II-A of Johnson and Francis [3]). Two non-sporeforming species, Eubacterium tenue and Peptostreptococcus anaerobius, also clustered within the confines of this group. The type strain of C. ghoni was found to be highly related to C. sordelli and E. tenue (98.5% and 99.0% sequence similarity, respectively). Clostridium bifermentans also displayed a relatively close relationship with these species (approx. 97%). Clostridium difficile and C. irregularis formed distinct lines within group II clostridia, with the latter species showing a specific association with C. lituseburensense and C. mayombeii. The type strain of C. villosum formed a relatively long branch and was only remotely related to other members of this group (Fig. 1).

The species C. aminovalericum, C. nexile and C. sphenoides formed a distinct phylogenetic cluster together with five other clostridia (viz. C. aminophilum, C. clostridiiforme, C. coccoides, C. oroticum and C. symbiosum) and the non-sporeforming species, 'Acetitomaculum ruminis' and Streptococcus hansenii (Fig. 1). With the exception of C. aminovalericum and 'A. ruminis', members of this grouping are characterised by an elevated DNA G + C content (approx. 38–52%). Two other species C. piliforme, causative agent of Tyzzer's disease [11], and the recently described 'giant' non-cultur able bacterium (Epulopiscium sp. [14]), displayed a peripheral affinity to this grouping.

Clostridium spiroforme was found to be genetically closely related to C. ramosum (96.1% 16S rRNA sequence similarity). Although it was evident from the treeing program that these two species form a distinct phylogenetic grouping, a specific albeit loose association was shown with several other sporeforming (C. innocuum) and non-sporeforming taxa (Erysipelothrix, Eubacterium biforme, Lactobacillus catenaformis and L. vitulinus) (Fig. 1).

The results of the present and earlier molecular taxonomic analysis [3,5–12] clearly show that the clostridia form a phylogenetically very diverse group of organisms. It is generally, if not universally, recognised that a profound revision of the classification of clostridia is now inevitable [1,2]. Full 16S rRNA sequencing studies demonstrate rRNA homology group I of Johnson and Francis
Fig. 1. Neighbour-joining dendrogram of small-subunit rRNA sequences showing the interrelationships of clostridial species and some non-sporeforming relatives. The dendrogram is based on comparison of approx. 1325 nucleotides (ranging from positions 103 to 1428, E. coli numbering system). The evolutionary distance between two species is the sum of the horizontal lengths between them.
[3] is a phylogenetically distinct grouping. Consequently, the genus *Clostridium* could be retained for organisms of this group, which embraces the type species *C. butyricum* (Fig. 1). The taxonomic treatment of the remaining rRNA groupings, however, will be more problematic. It is now firmly established that many non-sporeforming taxa are phylogenetically intertwined with clostridia. Consequently, the elevation of some clostridial rRNA groups into different genera will necessitate inclusion of their non-sporeforming relatives. The division of clostridia into different genera will require suitable characteristics to allow their clear phenotypic circumscription and differentiation. Although this may be possible for some minor groups (e.g. *C. ramosum/C. spiroforme*), no obvious solution seems forthcoming for those groups encompassing non-sporeforming species (e.g. *C. lituseburense* group) [2]. Progress in the taxonomic revision of clostridia is further compounded by the fact that some non-sporeforming genera (e.g. *Eubacterium*, *Peptostreptococcus*) are themselves phylogenetically extremely heterogeneous. Comprehensive sequence data are required for these taxa before the true phylogenetic structure of the clostridia and non-sporeforming relatives can be realised, and criteria to assist in the taxonomic recognition of the various rRNA groups sought.

**Acknowledgements**

This work was supported by grants from the Ministry of Agriculture, Fisheries and Food, and the EEC (HRAMI project, BIOT-CT91-0294 SSMA).

**References**


