16S rDNA analysis reveals phylogenetic diversity among the polysaccharolytic clostridia

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(Received 21 July 1993; accepted 27 July 1993)

Abstract: Small subunit rDNA sequences were determined for 13 mesophilic, polysaccharolytic, mainly cellulolytic species of the genus Clostridium and one cellulolytic Eubacterium species. Sequences were compared to those of 36 representatives of mesophilic and thermophilic clostridia, including those of nine thermophilic polysaccharolytic species published previously. The majority of strains group with 23S rRNA clusters I and III, while the others group with the thermophilic polysaccharolytic clostridia, i.e. C. stercorarium, C. thermolacticum and C. thermocellum. Lack of close genetic relationships between the various polysaccharolytic species is unexpected and may indicate that these biotechnologically important organisms differ with respect to the enzymology of polysaccharolytic degradation as well.

Key words: 16S rDNA; Systematics; Phylogeny; Polysaccharolytic; Clostridia

Introduction

The biotechnological potential of polysaccharolytic enzymes has resulted in the isolation and characterization of a large number of anaerobic, Gram-positive, spore-forming bacteria, the majority of which have been allocated to the genus Clostridium. In contrast to the thoroughly investigated mesophilic and thermophilic non-polysaccharolytic members of Clostridium, the polysaccharide degrading species of this genus have received less attention with respect to their genetic relationships. The previously investigated thermophilic, cellulolytic clostridia were found to be phylogenetically unrelated to the mesophilic clostridia [1]. It was the goal of this study to determine the degree of phylogenetic diversity and relationships among the mesophilic, polysaccharolytic clostridia. The information obtained from this study provides a basis for future reclassification of the clostridia as well as aiding in the selection of unrelated strains for further biotechnological investigations.

Materials and Methods

Strains investigated, together with their source, are shown in Table 1. Extraction of genomic DNA from lyophilized cell mass and amplifica-
Strains investigated and their source. All strains are type strains

<table>
<thead>
<tr>
<th>Organism</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. aldrichii</td>
<td>DSM 6159</td>
</tr>
<tr>
<td>C. celerecrescens</td>
<td>DSM 5628</td>
</tr>
<tr>
<td>C. cellubiovarum</td>
<td>DSM 1351</td>
</tr>
<tr>
<td>C. cellubiovarum</td>
<td>ATCC 35319</td>
</tr>
<tr>
<td>C. cellulosolvens</td>
<td>DSM 3052</td>
</tr>
<tr>
<td>C. chartatabidum</td>
<td>DSM 5482</td>
</tr>
<tr>
<td>C. lentocellum</td>
<td>DSM 5427</td>
</tr>
<tr>
<td>C. papyrosolvens</td>
<td>DSM 2782</td>
</tr>
<tr>
<td>C. polysaccharolyticum</td>
<td>DSM 1801</td>
</tr>
<tr>
<td>C. populeti</td>
<td>ATCC 35295</td>
</tr>
<tr>
<td>C. punicum</td>
<td>DSM 2619</td>
</tr>
<tr>
<td>C. termitidis</td>
<td>DSM 5396</td>
</tr>
<tr>
<td>C. xylanolyticum</td>
<td>ATCC 49623</td>
</tr>
<tr>
<td>E. cellulosolvens</td>
<td>ATCC 43171</td>
</tr>
</tbody>
</table>

PCR products were purified using the Prep-A-Gene Kit (Bio-Rad, USA) as described by the manufacturer. The Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, USA) was used to directly sequence the PCR products, following the protocol provided by the manufacturer. Electrophoresis of sequence reactions was done using the Applied Biosystems 373A DNA Sequencer. The sequences are available from EMBL under the accession numbers X71846–X71858 and X71860.

Small subunit 16S rDNA sequences were aligned against those of a set of 100 or so representatives of the Clostridium/Bacillus subphylum ([1,3] and recent database releases). Pairwise evolutionary distances were computed using the cor-

![Fig. 1. Phylogenetic position of mesophilic polysaccharolytic clostridia within the radiation of members of the genus Clostridium and its related taxa. The numbers of the DNA/23S rRNA clusters [6] are indicated I, II, III. B, Bacillus; C, Caldocellum; Cl, Clostridium; E, Eubacterium; Ep, Epulopiscium; T, Thermoaerobacterium; Tb, Thermoanaerobacter. Names in parentheses indicate that they are not validly published. The scale bar indicates 10 substitutions per hundred nucleotides.](https://academic.oup.com/femsle/article-abstract/113/2/125/512751)
rection of Jukes and Cantor [4]. Neighbour joining analysis was done according to Saitou and Nei [5]. In a subsequent analysis, the set of reference organisms was reduced to about 35 sequences, covering members of the major groups of the genus Clostridium and its phylogenetic relatives.

Results and Discussion

The 16S rDNA of fourteen polysaccharolytic, mainly cellulolytic species was sequenced almost completely (> 98% of the E. coli 16S rRNA sequence). C. cellulolyticum was found to have a 156 nucleotide long insertion in variable region 1, position 80. Similar long insertions in this region have been found in unrelated thermophilic clostridia, e.g. Thermoanaerobacterium xylanolyticum and Thermoanaerobacter ethanolicus [1]. More than 1100 unambiguously alignable nucleotides were analysed by evolutionary distances. A 50-by-50 similarity matrix was computed and is available upon request from the corresponding author.

The phylogenetic tree (Fig. 1), shows that three and six species sequenced fell into clusters I and III, respectively, as defined by DNA/23S rRNA similarity studies [6], while the other five species group with thermophilic and cellulolytic representatives of the genus Clostridium, i.e. C. stercolarum, C. thermolacticum and C. thermocellum. Bootstrap values (data not shown) indicate that the branching order of the ten or so major lines of descent have no statistical significance.

The internal structure of groups I and III are as follows:

The pectinolytic, amylolytic species C. punci
cenum is closely related to the cellulolytic but non-amylo
ytic species C. chartatabidum (94.9% similarity). Both organisms are also closely related to C. butyricum and C. carnis (in the range 89.2–92.9% similarity). The other member of cluster I, C. cellulovorans, hydrolysing cellulose, pectin and xylan, is only distantly related to other members of this cluster. It appears to share a common origin with C. thermopalmarium. Since C. butyricum, the type species of the genus, is a member of cluster I, this phylogenetically coher-
ent cluster can be considered the authentic genus Clostridium.

Within cluster III, the polysaccharolytic strains fell into three distinct subclusters. Despite distinct differences in DNA composition, C. polysaccharolyticum (42 mol%) and C. popauleti (28 mol%), two species that degrade a wide range of polysaccharides, are specifically related but not closely so (91.4% similarity). Both species cluster with C. aminovalericum (89.5–90.4% similarity). The closely related pair C. celerecrescens and C. xylanolyticum (97.5% similarity) differ in substrate range in that the latter species has not been shown to hydrolyse cellulose. Their phylogenetic neighbours are the misclassified Streptococcus hansenii and Eubacterium cellulosolvens (the type species of these two genera are genetically unrelated) and C. clostridiiforme. C. lentocellum stands isolated and branches intermediate to the causative agent of Tyzzers disease, C. piliforme, and the giant bacterium “Epulopiscium fishelsoni” recently described for an unculturable symbiont of surgeonfish [7] (87.5 and 83.9% similarity, respectively).

The remaining polysaccharolytic species form a phylogenetically coherent cluster, in which C. termmitidis, C. cellobiparum, C. cellulolyticum and C. papyrosolvens are highly related (96.5–99.1% similarity). Whether or not these species are actually strains of a single species needs to be determined by DNA/DNA similarity studies. Together with the other four deeper branching members, this subline (Group E according to Rainey et al. [1]) is so far solely defined by cellulolytic species. Other cellulolytic species of Clostridium are thermophilic, e.g. C. cellulosi and C. thermocopiace, organisms that are distantly related to the mesophilic cellulolytic members of this genus.

In conclusion, the traditional, phenotype-based distinction between proteolytic and saccharolytic members of Clostridium has no phylogenetic basis. Members of both metabolic types are intermixed as are mesophilic and thermophilic polysaccharolytic species. Our data reinforce the need to initiate a major revision of the phylogenetically heterogeneous genus as originally found by Johnson and Francis [6], later supported by
16S rRNA cataloguing data [8,9] and recent 16S rDNA studies [1], the wide range DNA mol% G + C (21–54 mol%) and the presence of spore- and non-sporeforming organisms within the radiation of clostridia. The low degree of relationships between polysaccharolytic species may stimulate selective studies on enzymes involved in the degradation of cellulose, xylan, starch and the like. This study provides the basis for the selection of genetically unrelated species for further biotechnological application.

Acknowledgement

This work was supported in part by a grant from the Australian Research Council to E.S.

References