Phylogenomic analyses of clostridia and identification of novel protein signatures that are specific to the genus *Clostridium sensu stricto* (cluster I)

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The species of *Clostridium* comprise a very heterogeneous assemblage of bacteria that do not form a phylogenetically coherent group. It has been proposed previously that only a subset of the species of *Clostridium* that form a distinct cluster in the 16S rRNA tree (cluster I) should be regarded as the true representatives of the genus *Clostridium* (i.e. *Clostridium sensu stricto*). However, this cluster is presently defined only in phylogenetic terms, and no biochemical, molecular or phenotypic characteristic is known that is unique to species from this cluster. We report here phylogenomic and comparative analyses based on sequenced clostridial genomes in an attempt to bridge this gap and to clarify the evolutionary relationships among species of clostridia. In phylogenetic trees for species of clostridia based on concatenated sequences for 37 highly conserved proteins, the species of *Clostridium* cluster I formed a strongly supported clade that was separated from all other clostridia by a long branch. Several other *Clostridium* species that are not part of this cluster grouped reliably with other species of clostridia in a number of well-resolved clades. Our comparative genomic analyses have identified three conserved indels in three highly conserved proteins (a 4 aa insert in DNA gyrase A, a 1 aa deletion in ATP synthase beta subunit and a 1 aa insert in ribosomal protein S2) that are unique to the species of *Clostridium* cluster I and are not found in any other bacteria. BLASTP searches on various proteins in the genomes of *Clostridium tetani* E88 and *Clostridium perfringens* SM101 have also identified more than 10 proteins that are found uniquely in the cluster I species. These results provide evidence that the species of *Clostridium* cluster I not only are phylogenetically distinct but also share many unique molecular characteristics. These newly identified molecular markers provide useful tools to define and circumscribe the genus *Clostridium sensu stricto* in more definitive terms. We have also identified a 7–9 aa conserved insert in the enzyme phosphoglycerate dehydrogenase that is uniquely found in the *Clostridium thermocellum*, *Thermoanaerobacter pseudethanolicus*, *Thermoanaerobacter tengcongensis* and *Caldicellulosiruptor saccharolyticus* homologues, and is absent from all other bacteria. These species form a well-defined clade in the phylogenetic trees and this indel provides a potential molecular marker for this clostridial cluster.

INTRODUCTION

The species belonging to the genus *Clostridium* comprise a heterogeneous assemblage that does not form a monophyletic grouping in phylogenetic trees based on different gene/protein sequences (Cato *et al.*, 1986; Lawson *et al.*, 1993; Stackebrandt *et al.*, 1999; Finegold *et al.*, 2002;

Wiegel *et al.*, 2006). Many species were originally assigned to this genus based on their being Gram-positive, anaerobic rods that are capable of endospore formation (Cato *et al.*, 1986; Cato & Stackebrandt, 1989; Lawson *et al.*, 1993; Finegold *et al.*, 2002; Rood, 2006). However, these criteria led to the placement of numerous species in this genus, many of which were very distantly related to the type species of the genus, *Clostridium butyricum* (Johnson & Francis, 1975; Cato *et al.*, 1986; Lawson *et al.*, 1993; Stackebrandt *et al.*, 1999; Wiegel *et al.*, 2006). Earlier work by a number of scientists, particularly by Collins, Stackebrandt and their co-workers, based on 16S rRNA gene sequences, suggested a number of important changes

Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

Details of proteins and strains used in the analyses and proteins found uniquely in either *C. perfringens* or *C. tetani* E88 and an NJ tree of the set II sequences are available as supplementary material with the online version of this paper.

to the taxonomy of the clostridia (Lawson *et al.*, 1993; Collins *et al.*, 1994; Stackebrandt *et al.*, 1999; Wiegel *et al.*, 2006). These studies led to the transfer of many previously known *Clostridium* species to new genera within the family *Clostridiaceae* and also to new families within the *Firmicutes* (Lawson *et al.*, 1993; Collins *et al.*, 1994; Stackebrandt *et al.*, 1999; Finegold *et al.*, 2002; Wiegel *et al.*, 2006). Based on the branching pattern of the clostridium species in the 16S rRNA gene tree, Collins and co-workers proposed that only a subset of the presently known *Clostridium* species, which form a distinct clade in the tree (cluster I), should be considered as true representatives of the genus *Clostridium* (i.e. *Clostridium sensu stricto*), and that other *Clostridium* species that lay outside this cluster should not be included in the genus *Clostridium* (Lawson *et al.*, 1993; Collins *et al.*, 1994). Of the current >150 *Clostridium* species with validly published names (see <http://www.bacterio.cict.fr>), fewer than half are part of cluster I (Wiegel *et al.*, 2006).

Although the proposal by Collins *et al.* (1994) to restrict the definition of the genus *Clostridium* to cluster I is an important step in terms of clarifying its taxonomy, cluster I also contains some members of other genera (e.g. *Anaerobacter*, *Eubacterium* and *Sarcina*) (Finegold *et al.*, 2002; Wiegel *et al.*, 2006; Rood, 2006). Moreover, this cluster is presently defined only in phylogenetic terms, and no biochemical, molecular or phenotypic characteristic is known that is unique to members of this cluster. Therefore, in order to circumscribe and define the genus *Clostridium* in clearer and more definitive terms, it is important to identify molecular markers that are uniquely shared by the members of this clade. In recent years, genome sequences have become available (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>) for a large number of prokaryotic organisms, including >130 firmicutes, of which 15 are from *Clostridium* species (Nolling *et al.*, 2001; Shimizu *et al.*, 2002; Brüggemann *et al.*, 2003; Myers *et al.*, 2006; Sebaihia *et al.*, 2006, 2007; Bettegowda *et al.*, 2006; Seedorf *et al.*, 2008). Comparative analyses of these and other clostridial genomes provide an unprecedented resource for discovering novel molecular characteristics that are either limited to a given species or are uniquely shared by different members of well-defined phylogenetic clades. Our recent work in this area has identified a large number of conserved indels (i.e. inserts or deletions) and whole proteins that are restricted to many higher taxonomic groups within the *Bacteria* (e.g. *Alphaproteobacteria*, *Epsilonproteobacteria*, *Chlamydia*, *Actinobacteria*, *Cyanobacteria*, *Deinococcus-Thermus*, *Bacteroidetes*, etc.; Griffiths *et al.*, 2006; Gao *et al.*, 2006; Gupta, 2006; Gupta & Griffiths, 2006; Gupta & Lorenzini, 2007; Gupta & Mok, 2007). These newly discovered molecular markers provide valuable tools for biochemical, diagnostic, taxonomic and evolutionary studies on these bacteria (Koonin & Galperin, 1997; Binnewies *et al.*, 2006).

In the present work, we report phylogenetic analyses on various clostridial species whose genomes have been sequenced based on concatenated sequences for 37

conserved proteins (including 20 ribosomal proteins) that are found ubiquitously in bacteria. In accordance with earlier work, in these trees, the cluster I species were clearly distinguished from all other *Clostridium* species. We have also carried out comparative genomic and phylogenomic analyses to identify molecular markers that are uniquely shared by the members of the genus *Clostridium* or related species. These studies have identified a number of conserved indels in important proteins and also many whole proteins that are distinctive characteristics of the sequenced cluster I *Clostridium* species. One conserved indel that is specific for another well-resolved clostridial cluster (consisting of *Clostridium thermocellum*, *Thermoanaerobacter* species and *Caldicellulosiruptor saccharolyticus*) is also described. These molecular markers provide useful means of clarifying the taxonomy of clostridia and for circumscribing the genus *Clostridium sensu stricto* in more definitive terms.

METHODS

Phylogenetic analyses. Phylogenetic analyses were carried out based on concatenated sequence alignments for two datasets of proteins. The first of these datasets contained 37 proteins, which included 20 ribosomal proteins and 17 other highly conserved proteins, which are present in virtually all bacteria (see Supplementary Table S1, available in IJSEM Online, for a list of these proteins) (Harris *et al.*, 2003; Ciccarelli *et al.*, 2006). For this set of proteins (set I), sequences for 28 firmicute species, which included all of the sequenced clostridia except *Clostridium novyi* (where a few proteins were missing) were retrieved (for details see Supplementary Table S2). The second set (set II), which was a subset of set I, consisted of 11 proteins [alanine-tRNA synthetase, arginine-tRNA synthetase, elongation factor-G (EF-G), EF-Tu, EF-P, DNA gyrase A, Hsp70, RNA polymerase β subunit, SecY, FtsY and CdsA] that are found in all of the bacteria. For this set, sequences for 26 firmicute species, which included all of the sequenced clostridia, including *C. novyi*, were retrieved (Supplementary Table S2). For *Clostridium perfringens* and *Clostridium botulinum*, where sequence information for more than one strain is available, only one strain (*C. perfringens* SM101 and *C. botulinum* ATCC 19397) was chosen. Multiple sequence alignments for these proteins were created using the CLUSTAL_X 1.83 program (Jeanmougin *et al.*, 1998). The sequence alignments for each set of proteins were concatenated separately and then imported into Gblocks 0.91b to remove poorly aligned regions (Castresana, 2000). The resulting final sequence alignments for the set I and set II proteins, which respectively consisted of 9421 and 4715 amino acids, were employed for phylogenetic analyses; protein sequence accession numbers are given on the alignments. Neighbour-joining (NJ) trees based on 1000 bootstrap replicates of these alignments were constructed using the TREECON 1.3b program (Van de Peer & De Wachter, 1994). The maximum-likelihood (ML) analysis was carried out using the WAG+F model with gamma distribution of evolutionary rates with four categories using the TREEPUZZLE program with 10 000 puzzling steps (Schmidt *et al.*, 2002). Maximum-parsimony (MP) trees based on 1000 bootstrap replicates were computed using the MEGA 4.1 program (Tamura *et al.*, 2007).

Identification of conserved indels specific to the clostridia.

Multiple sequence alignments for these and many other proteins that have been created in our earlier work (Griffiths & Gupta, 2004; Gao & Gupta, 2005) were visually inspected to identify any conserved indel that was restricted to either all or specific groups of the clostridia.

Indels that were not flanked by conserved regions were not considered. The specificities of these indels for the clostridia were evaluated by detailed BLAST searches on short sequence segments containing the indel and its flanking conserved regions. Sequence information for all indels was compiled into signature files that are presented here. For *C. perfringens* and *C. botulinum*, sequence information is available for a number of strains. The indicated indels were present in all of the sequenced strains (*C. perfringens*: SM101, ATCC 13124^T and strain 13; *C. botulinum*: A strain ATCC 19397, A ATCC 3502, A strain Hall, A3 strain Loch Maree, B1 strain Okra and F strain Langeland), which have virtually identical sequences for these regions. In the alignment files, sequence information is presented for only one of the strains of each of these species (*C. perfringens* SM101 and *C. botulinum* A strain ATCC 3502).

Identification of lineage-specific proteins. To identify proteins that are specific to the cluster I species, BLASTP searches were carried out on each protein (or open reading frame) in the genomes of *Clostridium tetani* E88 (Brüggemann *et al.*, 2003) and *C. perfringens* SM101 (Myers *et al.*, 2006). These searches were carried out using the default parameters, without the low-complexity filter, to identify proteins where all significant hits were from this group of species (Altschul *et al.*, 1997). The proteins that were of interest generally involved a large increase in the expected values (E-values) from the last hit for the cluster I clostridia to the first hit from any other organism. Further, the E-values of these latter hits were generally greater than 10^{-3} , which indicates a weak level of similarity that can occur by chance (Gao *et al.*, 2006; Gupta, 2006; Gupta & Mok, 2007). All promising proteins were further analysed using the PSI-BLAST program (Schaffer *et al.*, 2001) to confirm their group specificity. For all of the identified proteins, their protein identification numbers in the *C. tetani* E88 or *C. perfringens* SM101 genome, accession numbers and information regarding possible functions are provided.

RESULTS

The evolutionary relationships among the clostridia have been studied in detail primarily based on 16S rRNA gene sequences (Lawson *et al.*, 1993; Collins *et al.*, 1994). The availability of large numbers of genome sequences, which include more than 20 from different clostridia representing several orders and families from this class of bacteria, now provides an opportunity to study their evolutionary relationships based on sequences for multiple conserved proteins. Phylogenetic analyses based on a large dataset of concatenated sequences for multiple proteins are more reliable than those based on any single gene or protein (Rokas *et al.*, 2003; Brown & Volker, 2004; Belda *et al.*, 2005; Ciccarelli *et al.*, 2006). We have carried out phylogenetic analyses for the clostridia based on two large datasets of protein sequences. The larger of these datasets (set I) consist of 37 proteins, which form the core of all prokaryotes (Harris *et al.*, 2003; Ciccarelli *et al.*, 2006). The second dataset of 11 proteins is a subset of the larger dataset. Phylogenetic analyses based on concatenated sequence alignments for these proteins were carried out using the NJ, MP and ML methods.

An NJ distance tree for the clostridia based on a concatenated sequence alignment for the 37 proteins (set I) is shown in Fig. 1. The bootstrap scores of various nodes in this tree using the NJ, MP and ML methods (>50 %) are shown. The results for set II, which also included *C. novyi*, were very similar (Supplementary Fig. S1). As seen, the

Fig. 1. NJ distance tree for the clostridia and some other firmicutes based on concatenated sequences for 37 conserved proteins. Bootstrap scores for various nodes in the NJ/MP/ML trees >50 % are indicated at nodes. The species of *Clostridium* cluster I as well as another cluster of clostridia that could be identified based on conserved indels are marked.

various *Clostridium* species did not form a monophyletic clade in these trees. A number of *Clostridium* species, including *C. tetani*, *C. botulinum*, *C. kluyveri*, *C. acetobutylicum*, *C. novyi*, *C. perfringens* and *C. beijerinckii*, formed a well-defined clade that was recovered in 100% of the bootstrap samples (or puzzling quartets in the ML tree) and it was separated from all other clostridia by a long branch. All of the species in this clade corresponds to *Clostridium sensu stricto* or cluster I, as previously identified by Lawson *et al.* (1993) and Wiegel *et al.* (2006). In the NJ tree shown here,

Clostridium phytofermentans formed an outgroup of the cluster I species. However, the low bootstrap score of this combined clade and the long branch that separated this species from all other cluster I species indicated that this affiliation was not reliable. In contrast to the NJ tree, in both the ML and MP trees, *C. phytofermentans* formed an outgroup of a clade comprising *Clostridium difficile* and the two *Alkaliphilus* species. Although the grouping of *C. difficile* with '*Alkaliphilus metalliredigens*' and '*Alkaliphilus oremlandii*' was strongly supported by different methods, the

Fig. 2. Partial sequence alignment of gyrase A homologues showing a 4 aa insert (boxed) in a conserved region that is found uniquely in all of the species of *Clostridium* cluster I for which a sequence was available. In contrast to other bacteria, the cluster I species have two different gyrase A homologues, and the identified insert is found in only one of them. Dashes (-) in this and all other sequence alignments denote identity with the amino acid on the top line. The position of the sequence in the *E. coli* protein is marked on the top. Sequence information for only representative species is presented. The indicated insert is not found in any other species.

affiliation of *C. phytofermentans* with this clade was not reliable (<50% bootstrap scores). Another clostridium species, *C. thermocellum*, grouped reliably with the two *Thermoanaerobacter* species (*Thermoanaerobacter pseudethanolicus* and *Thermoanaerobacter tengcongensis*) and *Caldicellulosiruptor saccharolyticus*. The above relationships, which were supported by trees based on both set I and set II sequences, confirm the inference from earlier studies that the *Clostridium* species are phylogenetically diverse and do not form a monophyletic group (Lawson *et al.*, 1993; Collins *et al.*, 1994; Stackebrandt *et al.*, 1999; Finegold *et al.*, 2002; Wiegel *et al.*, 2006). The distinct branching of the cluster I species and the long branch that separated them from all other clostridia also support the proposal to recognize this

group of species, which includes the majority of the medically important species, as the genus *Clostridium sensu stricto* (Lawson *et al.*, 1993; Collins *et al.*, 1994; Wiegel *et al.*, 2006).

Conserved indels in protein sequences that are specific for cluster I and other subgroups of the clostridia

Rare genomic changes such as conserved inserts and deletions in genes/proteins that are restricted to species from phylogenetically well-defined groups provide powerful means of confirming evolutionary relationships among species as well as valuable molecular markers for taxonomic, genetic and biochemical studies (Rivera & Lake,

		209	258	
Clostridium cluster I	<i>Clostridium acetobutylicum</i>	15896119	VFGQMNEPPGARMRVALTGLTMAEYFRD	KGQDVLFLFDINIFRFTQAGSEV
	<i>Clostridium perfringens</i>	YP_699462	-----K-----	Q-----S-----
	<i>Clostridium pasteurianum</i>	14715606	-----K-----	E-----
	<i>Clostridium beijerinckii</i>	YP_001307562	-----H-----	-----Y-----
	<i>Clostridium kluyveri</i>	ZP_01840201	-----H-----	E-----
	<i>Clostridium novyi</i>	YP_878996	-----S-----	Q-N-----
	<i>Clostridium botulinum</i>	YP_001252703	-----S-----	Q-----
	<i>Clostridium tetani</i>	NP_782272	-ICATDK-ALV-IGK-F-ATAI-----	-KK-I-MM-SVT--AM-QR--
	<i>Clostridium paradoxum</i>	ABB13426.1	-Y-----	Q E-----
	<i>Clostridium phytofermentans</i>	ZP_01354913	-----G-----	K S-K-----
Other clostridia	<i>Clostridium difficile</i>	YP_001089988	-----H-----	E Q-----V-----
	<i>Clostridium cellulolyticum</i>	ZP_01575505	-----G-----	Q M-----V-----
	<i>Clostridium thermocellum</i>	23021351	-----S---G-----	E L-----I-----
	<i>Alkaliphilus metalliredigens</i>	YP_001318242	-Y-----G-----	Q E-----
	<i>Ruminococcus obeum</i>	EDM89195	-Y-----G-S-----	E-N-----
	<i>Desulfotobacterium hafniense</i>	23114962	-----L-G-----	V QN-----
	<i>Ruminococcus albus</i>	2662066	-Y-----G-S-----	V E-----
	<i>Thermoanaerobac. tengcongensis</i>	20807130	-----G-A---I-----	E EH-----V-----
	<i>Streptococcus agalactiae</i>	22537026	-----I-----	V E-----
	<i>Streptococcus sanguinis</i>	4100657	-----I-----	V E-----
Other firmicutes	<i>Streptococcus pyogenes</i>	19745847	-----I-----	V E-----
	<i>Streptococcus pneumoniae</i>	18253303	-----I-----	V E-----
	<i>Streptococcus mutans</i>	4239652	-----I-----	V E-----
	<i>Streptococcus thermophilus</i>	20070093	-----I-----	V E-----
	<i>Lactobacillus acidophilus</i>	6652833	-----L-----	V E-----
	<i>Lactobacillus casei</i>	416679	-----I-----	V E-----
	<i>Lactobacillus gasseri</i>	23002570	-----I-----	V E-L-----
	<i>Lactococcus lactis</i>	22653666	-----S-----	E E-----
	<i>Staphylococcus epidermidis</i>	27468618	-----S-----	E E-----
	<i>Lactobacillus plantarum</i>	28378938	-Y-----I-----	V Q-----
	<i>Leuconostoc mesenteroides</i>	23023845	-Y-----N-----	N E-K-----
	<i>Mycoplasma gallisepticum</i>	31544510	-----V NN-----	V NN-----
	<i>Mycoplasma pulmonis</i>	15828737	-----K FK-----	K FK-----
	<i>Mycoplasma pneumoniae</i>	13508337	-----A-----	H DN-----
	<i>Ureaplasma urealyticum</i>	13357686	-----S-----E-----	A F-----
	<i>Listeria innocua</i>	16801734	-----I-----	E EH-----
	<i>Listeria monocytogenes</i>	16804567	-----I-----	E EH-----
	<i>Bacillus caldotenax</i>	728922	-----H-----	E Q-----
	<i>Bacillus subtilis</i>	16080734	-----S-----H-----	V Q-----F-----
	<i>Bacillus halodurans</i>	15616316	-----S-----H-----	K Q-----
<i>Bacillus pseudofirmus</i>	14916961	-----S-----H-----	R D-----V-----	
Other bacteria	<i>Bacillus anthracis</i>	21397777	-----Q-----H-----	E Q-----
	<i>Bacillus cereus</i>	30023337	-----Q-----H-----	E Q-----
	<i>Staphylococcus aureus</i>	15925093	-----S-----	E Q-----
	<i>Geobacillus thermoleovorans</i>	7578511	-----G-----D-----	Q E-K-----
	<i>Oenococcus oeni</i>	21684964	-----P-----	E Q-----N-----
	<i>Geobacillus stearothermophilus</i>	1168569	-----P-----	E Q-----V-----
	<i>Oceanobacillus iheyensis</i>	23100430	-----I-----	V E-----
	<i>Enterococcus faecium</i>	22991789	-----I-----	V E-----
	<i>Enterococcus faecalis</i>	29377093	-----I-----	V E-----
	<i>Pediococcus damnosus</i>	28625013	-Y-----I-----	V E-----
	<i>Pediococcus parvulus</i>	28625015	-Y-----L-----	V E-----
	<i>Corynebacterium glutamicum</i>	2118190	-----D---V---S-----	V QN-----
	<i>Corynebacterium efficiens</i>	25027871	-----D---V---S-----	V QN-----
	<i>Mycobacterium tuberculosis</i>	15608450	-----D---T---SA---W-----	E Q-----
	<i>Streptomyces coelicolor</i>	21223733	-----D---T-L---A-----	V QK---F-----
	<i>Streptomyces avermitilis</i>	29829423	-----D---T-L---A-----	V QK---F-----
	<i>Tropheryma whippelii</i>	28493391	-----D---T-L---SA-----	V QK---F-----
	<i>Thermobifida fusca</i>	23018730	-----D---T-L---SA-----	V QK---F-----
	<i>Fervidobacterium islandicum</i>	3913123	-----F---A---I-----	V E-R-----V-----
	<i>Thermotoga neapolitana</i>	24298790	-----F---A---I-----	V E-R-----V-----
	<i>Thermotoga maritima</i>	15644358	-----F---A---I-----	V E-R-----V-----
	<i>Fusobacterium nucleatum</i>	19703700	-Y-----L-----V-N-----	K D-----

Fig. 3. Partial sequence alignment of ATP synthase beta subunit homologues showing a 1 aa deletion (corresponding region in other species is boxed) that is found uniquely in all of the species of *Clostridium* cluster I for which a sequence was available, but not found in any other bacteria.

1992; Gupta, 1998; Rokas & Holland, 2000). We have identified a number of conserved indels in important proteins that are helpful in defining and circumscribing a number of clades of the clostridia, including cluster I.

The DNA topoisomerases play an essential role in DNA replication and transcription processes by maintaining appropriate levels of supercoiling in chromosomal DNA (Levine *et al.*, 1998; Corbett & Berger, 2004). Of these, the type II isomerases make transient double-strand breaks in the DNA to allow another strand to pass through it. Our sequence alignment and BLAST searches on the gyrase A protein have revealed that all sequenced cluster I *Clostridium* species (*C. tetani*, *C. botulinum*, *C. kluyveri*, *C. acetobutylicum*, *C. novyi*, *C. perfringens* and *C. beijerinckii*) contain two different homologues of the protein. A partial sequence alignment of these proteins from various clostridia, firmicutes and some other bacteria is presented in Fig. 2. In one of these homologues (which is variously annotated as GyrA, GyrA2, DNA topoisomerase

IV subunit A and ParC), all species of *Clostridium* cluster I contain a 4 aa insert in a highly conserved region that is not found in any other clostridia or other groups of bacteria. Detailed BLAST searches with the insert-containing homologue confirm that this insert is a distinctive characteristic of the species of *Clostridium* cluster I. Two different kinds of type II DNA topoisomerases (DNA gyrase and Topo IV), which carry out similar but not identical functions (Levine *et al.*, 1998; Corbett & Berger, 2004), have been identified in some bacteria. BLAST searches with either the insert-containing or the insert-lacking homologues show that the top 100 hits in both cases all correspond to the gyrase A subunit from various bacteria. These results indicate that both of these homologues correspond to the gyrase A subunit, although the possibility that one of them (i.e. insert-containing) could be involved in Topo IV function cannot be excluded.

Two other conserved indels that are specific for cluster I of *Clostridium* are present in the proteins ATP synthase beta

		129		187
Clostridium cluster I	<i>Clostridium acetobutylicum</i>	15895063	LFPKKEVILRNNEEEKLVKNLGGIVNMTQSN	I GALFVVDPRKEKNAISEAKILGIPVVAI
	<i>Clostridium novyi</i>	118442952	-----K-----K-----E-----K-LDA--	L --I-I-----D--N-----
	<i>Clostridium kluyveri</i>	149092096	-S-----K-----KQ--ER-----KA-NAE--	V --M-I-----L-----
	<i>Clostridium beijerinckii</i>	150016072	-----K-KG-M-----E-----R-LDA--	V --M-I-----L-----
	<i>Clostridium perfringens</i>	YP_698987	-----K-KL-R-----QR-----KELDA--	I --M-----A-----
	<i>Clostridium botulinum</i>	YP_001254934	-----K-K-R-----E-----RKLDAN--	V --M-I-----L--R-----
	<i>Clostridium tetani</i>	28210947	-----K-KL-----E-----K-LNAN--	V --M-----L-----
	<i>Clostridium</i> sp. OhILAs	106894610	-----K--H-Q-----E-F-----KD--EMP	D-----RI--Q--HT-----IS
	<i>Clostridium thermocellum</i>	125973521	-----TK--A-K-----E-Y-----KE-KELP	-----RI-VL--RR-----
	<i>Clostridium difficile</i>	126699757	-----K--A-K-----E-Y-----KD-PELP	--M-----NI--Q--HR-----G
Other clostridia	<i>Clostridium cellulolyticum</i>	118726999	-----SK-KK-MAD-E-----K--KKLP	--M-----R--L--R-----
	<i>Clostridium phytofermentans</i>	106885079	-----E-KK-W-----E-----KD-KEVP	E-I--K--RICQ-Q--HN--VKLIG
	<i>Thermosinus carboxydivorans</i>	121533730	-----MA--H-M-R-Q-F-----K--NKLP	-----II-----RI-VA--RK-----I
	<i>Alkaliphilus metalliredigens</i>	149950313	-----K--H-M-----E-F-----KG--MP	D--I-----RI--K--QT-----IS
	<i>Desulfotomaculum reducens</i>	134299825	-----AQ-LH-K-----D-F-----K--RRLP	-----I-----RI-VA--RK-----I
	<i>Thermoanaerobacterium ethanolicum</i>	114843693	-----K--K-K-R-Q-F-----E--QSLP	S--I--K--AI-VA--RS-E--I--
	<i>Thermoanaerobacterium tengcongensis</i>	20807856	-----R--K-K-R-Q-F-----E--ESLP	S--I--K--AI-V--RA-E--I--
	<i>Syntrophomonas aciditrophicus</i>	85859430	F---IMG-QK-R-----V-----QK-KSLP	A--I--K--TI-VN--K--R--II--
	<i>Anaeromyxobacter dehalogenans</i>	86156701	-----AQ-ER-R-----E-----KELSLP	S--I--TK--HI-VH--NR-----V
	<i>Ruminococcus torques</i>	145846182	-----E-KK-WA-----E-----KD-KRLP	D-I-I--K--RICVQ-Q--HT-----LIG
Other firmicutes	<i>Ruminococcus obeum</i>	149833320	-----E--K-LA--Q-----E-----KE-KRLP	D-I-I--K--RICVQ-Q--HT-----LIG
	<i>Moorella thermoacetica</i>	83589880	-----AH--G-R-----ERF-----KE-KSLP	D--II-----RI-VA--GRR-----I
	<i>Dorea longicatena</i>	149751984	-----Q-KK-WD-----E-----KD-KTLP	D-I-I--K--RICVQ-Q--HT-----LIG
	<i>Syntrophomonas wolfei</i>	114566417	-S-----AR-L--R-R-ERF-----KE-DKLP	--V-----RI-VA--RK-N-----
	<i>Desulfitobacterium hafniense</i>	89895292	-----AA--H-M-----ERF-----KS-KKLP	D-----RI-VA--RR-N--I-G
	<i>Caldicellulosum saccharolyticum</i>	146297357	-----NL--K-K-R-L-Y-----Q--PRIP	DI-YI-----R-VL--RK-----I
	<i>Peloto. thermopropionicum</i>	147677587	-----AE-MH-K-----Q-F-----KE-RRLP	-----I-----RI-VA--RK-----I
	<i>Carboxydo. hydrogenoforman</i>	78042772	-----AKILK-K--SRF-S--KE-KKLP	D-----I-----VA--RK-D-----
	<i>Staphylococcus aureus</i>	57651825	-----VE-KK-YDR-I-F-----RD-KSMP	Q-----R--A--RK-N--I-G
	<i>Staphylococcus haemolyticus</i>	70726659	-----VE-KK-YDR-I-F-----RD-KSMP	Q-----R--A--RK-N--I-G

Fig. 4. Partial sequence alignment of ribosomal protein S2 homologues showing a 1 aa insert (boxed) that is specific for the species of *Clostridium* cluster I for which a sequence was available and not found in any other bacteria. Sequence information for only representative species is presented.

subunit (Fig. 3) and ribosomal protein S2 (Fig. 4). In the former case, a 1 aa deletion in a highly conserved region is present in all of the cluster I species but it is not found in any of the other *Clostridium* species (*Clostridium paradoxum*, *C. phytofermentans* ISDg^T, *C. difficile* 630, *C. cellulolyticum* and *C. thermocellum* ATCC 27405^T) that do not form part of this cluster. This indel is also not found in any other bacteria, indicating that it is a deletion in the cluster I species. Similarly, in the ribosomal protein S2, a 1 aa insert is present in a conserved region in all cluster I *Clostridium* species, but it is not found in any of the other *Clostridium* species (*Clostridium* sp. OhILAs, *C. phytofermentans*, *C. difficile* 630, *C. cellulolyticum*, *C. thermocellum*) or other bacteria. Both of these indels are therefore distinctive characteristics of the cluster I species.

An additional conserved indel that serves to define and circumscribe another cluster of the clostridia has been identified in the enzyme phosphoglycerate dehydrogenase, which carries out the first and rate-limiting step in the phosphorylated pathway of serine biosynthesis (McKittrick & Pizer, 1980). In this case, a 7–9 aa insert is found uniquely in *C. thermocellum*, *T. pseudethanolicus*, *T. tengcogensis* and *Caldicellulosiruptor saccharolyticus* homologues, but is absent in all of the cluster I species, *C. difficile* and various other bacteria (Fig. 5). The group of species that contain this insert form a well-defined clade in phylogenetic trees based on both sets of concatenated

sequences (Fig. 1 and Supplementary Fig. S1). Thus, it is highly likely that this insert was introduced in a common ancestor of this clade of species, and it serves to define this clade or subgroup of clostridia in molecular terms.

Whole proteins that are specific for *Clostridium* and the cluster I species

We have also carried out systematic BLAST searches on various open reading frames in the genomes of *C. tetani* E88 and *C. perfringens* SM101 to identify proteins that are found uniquely in *Clostridium* species at various phylogenetic depths. These studies have identified many proteins that are found uniquely in *Clostridium* species. As expected, based on earlier work (Shimizu *et al.*, 2002; Brüggemann *et al.*, 2003; Myers *et al.*, 2006), a large number of proteins (151 for *C. perfringens* and 86 for *C. tetani*) are found only in these particular *Clostridium* species (Supplementary Table S3). For *C. perfringens*, of which multiple strains have been sequenced (Shimizu *et al.*, 2002; Myers *et al.*, 2006), most of these proteins are present in all sequenced strains. Fifteen additional proteins are found only in *C. perfringens* and *C. beijerinckii* and another group of 15 proteins are specific for *C. tetani* and *C. botulinum* (Table 1). In the phylogenetic trees based on concatenated proteins (Fig. 1 and Supplementary Fig. S1), *C. perfringens* and *C. beijerinckii* form a distinct clade that is supported by high

Fig. 5. Partial sequence alignment of phosphoglycerate dehydrogenase homologues showing a 7–9 aa insert that is only found in the homologues from *C. thermocellum*, *T. pseudethanolicus*, *T. tengcogensis* and *Caldicellulosiruptor saccharolyticus*. The insert in *C. thermocellum* contains three amino acids (KKP) at the position marked with *. The species containing this insert form a well-defined clade in the phylogenetic tree (Fig. 1) and this molecular signature appears to be a distinctive characteristic of this clade.

Table 1. Proteins unique to certain species within *Clostridium* cluster I

All significant BLASTP hits for these proteins are for the *Clostridium* species indicated.

Genome ID	NCBI protein accession no.
Proteins specific to <i>C. perfringens</i> and <i>C. beijerinckii</i>	
CPR_0643	YP_697970
CPR_1211	YP_698533
CPR_1369	YP_698689
CPR_1378	YP_698697
CPR_1393	YP_698712
CPR_1451	YP_698770
CPR_1468	YP_698787
CPR_1498	YP_698817
CPR_1503	YP_698822
CPR_1802	YP_699117
CPR_1926	YP_699236
CPR_1932	YP_699242
CPR_2321	YP_699587
CPR_2574	YP_699821
CPR_2634	YP_699871
Proteins specific to <i>C. tetani</i> E88 and <i>C. botulinum</i>	
CTC01928	NP_782502
CTC02107	NP_782668
CTC00547	NP_781230
CTC00615	NP_781291
CTC01032	NP_781681
CTC01093	NP_781738
CTC01095	NP_781740
CTC01096	NP_781741
CTC01097	NP_781742
CTC01101	NP_781745
CTC01102	NP_781746
CTC01104	NP_781748
CTC01105	NP_781749
CTC01167	NP_781803
CTC01696	NP_782294

(97–100%) bootstrap scores. Likewise, *C. tetani* and *C. botulinum* are also indicated as close relatives in these trees (Fig. 1 and Supplementary Fig. S1). The unique shared presence of these proteins in these species provides further evidence of their close evolutionary relationship.

Of particular interest in the present context is that we have identified 10 proteins that are found uniquely in all of the genome sequences from species of *Clostridium* cluster I (*C. tetani*, *C. botulinum*, *C. kluyveri*, *C. acetobutylicum*, *C. novyi*, *C. perfringens* and *C. beijerinckii*) (Table 2). No significant BLAST hit for these proteins was detected in any other bacteria. Nine additional proteins included in Table 2 are also specific for the cluster I species, but they are missing from one or two of the seven sequenced genomes.

Table 2. Proteins that are found uniquely in the species of *Clostridium* cluster I

Unless otherwise indicated, all significant hits in BLASTP searches for these proteins are from *Clostridium* cluster I species (i.e. genus *Clostridium sensu stricto*) and, unless otherwise noted, homologues of these proteins are present in all of the sequenced cluster I species (*C. tetani* E88, *C. kluyveri* DSM 555^T, *C. botulinum*, *C. perfringens*, *C. novyi* NT, *C. acetobutylicum* ATCC 824^T, *C. beijerinckii* NCIMB 8052^T). For *C. botulinum* and *C. perfringens*, most of these protein sequences are found in all sequenced strains (*C. perfringens*: SM101, ATCC 13124^T, strain 13; *C. botulinum*: A strain ATCC 19397, A ATCC 3502, A strain Hall, A3 strain Loch Maree, B1 strain Okra and F strain Langeland).

Genome ID	Accession no.	Possible function
CTC00252	NP_780961	Hypothetical
CTC00303	NP_781008	Membrane protein
CTC00970	NP_781622	Hypothetical
CTC00991	NP_781641	Hypothetical
CTC01016	NP_781665	Lipoprotein
CTC01297	NP_781926	Hypothetical
CTC01645	NP_782249	Hypothetical
CTC01740	NP_782335	Hypothetical
CTC02077	NP_782639	Hypothetical
CTC02157	NP_782713	Hypothetical
CTC00178 ^{a*}	NP_780892	Hypothetical
CTC0181 ^{ab}	NP_780895	Hypothetical
CTC00294 ^c	NP_781000	Hypothetical
CTC00345 ^d	NP_781047	Hypothetical
CTC01053 ^b	NP_781699	Hypothetical
CTC01597 ^{ac}	NP_782205	Hypothetical
CTC02136 ^{bc}	NP_782695	Hypothetical
CTC02138 ^b	NP_782697	Hypothetical
CTC02222 [†]	NP_782769	Hypothetical

*Protein missing from the genome sequence of the following species: a, *C. beijerinckii*; b, *C. novyi*; c, *C. perfringens*; d, *C. botulinum*.

†Significant hit also observed in *Solibacter usitatus*.

The species distribution of these proteins indicates that the genes for them probably evolved in a common ancestor of the species of *Clostridium* cluster I.

DISCUSSION

The results presented here confirm that the species of *Clostridium* cluster I form a phylogenetically cohesive group and that they are distinct from other *Clostridium* species that do not form part of this well-defined clade. Our comparative genomic analyses have also identified a large number of molecular characteristics, consisting of conserved indels and whole proteins, that are distinctive characteristics of the species of *Clostridium* cluster I. These newly identified molecular markers further confirm the distinctness of the species of *Clostridium* cluster I and they strongly support the proposal of recognizing only species

belonging to this cluster as true *Clostridium* species, i.e. *Clostridium sensu stricto*. Based upon these results, the species from this group are distinct not only in phylogenetic terms but also in molecular and, by inference, biochemical terms. These molecular markers now provide new tools to define and circumscribe the genus *Clostridium sensu stricto* in more definitive molecular terms.

Sequence information for these newly identified molecular characteristics is presently limited to only those species whose genomes have been sequenced. In order to confirm and validate the usefulness of these molecular markers as distinctive characteristics of the genus *Clostridium sensu stricto*, it will be necessary to obtain sequence information for them from both other clostridia belonging to cluster I and those that fall outside its radiation. It will be particularly interesting to obtain sequence information for these signatures for a number of species belonging to the other clostridial genera (e.g. *Anaerobacter polyendosporus*, *Eubacterium moniliforme*, *Eubacterium tarantellae*, *Sarcina ventriculi* and *Sarcina maxima*) that branch within cluster I (Wiegel *et al.*, 2006). The presence of these *Clostridium*-specific signatures in these species would provide a strong case for their transfer to the genus *Clostridium*. Because all of the conserved indels described here are found in highly conserved regions, it should be possible to obtain sequence information for these signatures from other species by using degenerate PCR primers flanking the indel-containing regions, as described in our other work (Griffiths *et al.*, 2005; Gao & Gupta, 2005).

The cellular functions of virtually all of the proteins that are specific either for a given *Clostridium* species or for cluster I are presently not known. In a few cases, a putative function has been ascribed. However, based on the fact that these proteins do not exhibit significant similarity to proteins from other species, it is highly likely that they may carry out entirely different cellular functions (Galperin & Koonin, 2004). For the proteins that are specific to *Clostridium* cluster I, their persistence in all sequenced species from this clade and generally high degree of sequence conservation strongly suggest that they play important functions within this group of bacteria (Fang *et al.*, 2005). Since cluster I includes many important human and animal pathogens, functional studies on these conserved proteins will be of much interest.

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