The use of signature sequences in different proteins to determine the relative branching order of bacterial divisions: evidence that *Fibrobacter* diverged at a similar time to *Chlamydia* and the *Cytophaga–Flavobacterium–Bacteroides* division

Emma Griffiths and Radhey S. Gupta

Author for correspondence: Radhey S. Gupta. Tel: +1 905 525 9140 ext. 22639. Fax: +1 905 522 9033.
e-mail: gupta@mcmaster.ca

Department of
Biochemistry, McMaster
University, Hamilton,
Ontario, Canada L8N 3Z5

The phylogenetic placement of the rumen bacterium *Fibrobacter succinogenes* was determined using a signature sequence approach that allows determination of the relative branching order of the major divisions among *Bacteria* [Gupta, R. S. (2000) *FEMS Microbiol Rev* 24, 367–402]. For this purpose, segments of the Hsp60 (*groEL*), Hsp70 (*dnaK*), CTP synthase and alanyl-tRNA synthetase genes, which are known to contain signature sequences that are useful for phylogenetic determinative purposes, were cloned. Using degenerate oligonucleotide primers for highly conserved regions in these proteins, 1.4 kb, 0.75 kb, 401 bp and 171 bp fragments of the Hsp70, Hsp60, CTP synthase and alanyl-tRNA synthetase genes respectively were amplified by PCR, and these fragments were cloned and sequenced. These primers, because of their high degree of conservation, could also be used for cloning these genes from other bacterial species. The Hsp70 homologues from different Gram-negative bacteria contain a 21–23 aa insert that is not found in any Gram-positive bacteria. The presence of this insert in the *F. succinogenes* Hsp70 supports its placement within the Gram-negative group of bacteria. A conserved insert in *F. succinogenes* Hsp60 that is commonly present in all bacterial species, except various Gram-positive bacteria, *Deinococcus–Thermus* groups and green non-sulphur bacteria, provides evidence that *F. succinogenes* does not belong to these taxa. A particularly useful signature consisting of a 4 aa insert is found in Ala-tRNA synthetase. This insert is present in all proteobacterial homologues as well as in homologues from species belonging to the *Chlamydia* and *Cytophaga–Flavobacterium–Bacteroides* (CFB) groups, but it is not found in homologues from any other groups of bacteria. The presence of this insert in *F. succinogenes* Ala-tRNA synthetase provides evidence that this species is related to these groups. However, two other signatures in CTP synthase and Hsp70 proteins, that are distinctive of the proteobacterial species, are not present in the *F. succinogenes* homologues. These results provide evidence that *F. succinogenes* does not belong to the proteobacterial division and thus should be placed in a similar position as the *Chlamydia* and CFB groups of species.

Keywords: bacterial evolution, phylogeny, phylogenetic placement, *Fibrobacter succinogenes*, fibrolytic bacteria

Abbreviation: CFB, *Cytophaga–Flavobacterium–Bacteroides*.

The GenBank accession numbers for the sequences reported in this paper are AY017380, AY017381, AY017382 and AY017383.

INTRODUCTION

The rumen bacterium *Fibrobacter succinogenes* contains a number of cellulolytic and hemicellulolytic enzymes which are responsible for the digestion of the cell walls of forage plants in the bovine rumen (Cheng *et al.*, 1991; Gokarn *et al.*, 1997). Because of the established role of *F. succinogenes* as one of the major indigenous fibrolytic micro-organisms in ruminants, there is much interest in this species from the viewpoints of farm production efficiency, alternative waste management, and its evolutionary relationship to other bacteria (Asanuma *et al.*, 1999; Cheng *et al.*, 1991; Debroas & Blanchart, 1993; Fields *et al.*, 2000; Garcia-Vallve *et al.*, 2000; Huang & Forsberg, 1990; Shi *et al.*, 1997; Wang *et al.*, 2000; Weimer *et al.*, 1999).

Based on its physiological characteristics, *F. succinogenes*, originally known as *Bacteroides succinogenes*, was previously considered to be a member of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) division (Hungate, 1950). However, subsequent studies based on 16S rRNA indicated that this and other related strains were not closely related to other species of *Bacteroides*, leading to their placement in a separate genus named *Fibrobacter* (Montgomery *et al.*, 1988). A recent taxonomic classification based on 16S rRNA places *Fibrobacter* in a separate division, distinct from the CFB group, originating from the common bacterial ancestor (Amann *et al.*, 1992; Maidak *et al.*, 1999; Ludwig & Schleifer, 1999; Zinder, 1998). Our current understanding of the evolutionary relationships among prokaryotes is based almost entirely on 16S rRNA sequences (Hugenholtz *et al.*, 1998; Maidak *et al.*, 1999; Olsen & Woese, 1993; Woese, 1987). Although, the phylogenetic trees based on 16S rRNA have proven extremely useful in the placement of species into different groups, one of their main recognized limitations has been their inability to resolve the relationships among the major divisions within bacteria or to deduce how these groups branched from a common ancestor (Maidak *et al.*, 1999; Ludwig & Schleifer, 1999; Gupta, 1998a, 2000c). Recently, we have described a new approach based on the presence of shared conserved indels (or signature sequences) in different proteins for deducing the phylogenetic relationships among different bacterial groups, that complements this major deficiency of the rRNA trees (Gupta, 1998a, 2000a). By following the presence or absence of signature sequences in a number of different proteins in various divisions of bacteria, it is now possible to deduce logically how the major taxa within the *Bacteria* have branched from a common ancestor. The branching order for various divisions as determined from these studies is as follows: low-G + C Gram-positives → high-G + C Gram-positives → *Deinococcus-Thermus* → green non-sulphur bacteria → cyanobacteria → spirochaetes → *Chlamydia*-CFB group → δ,ϵ -*Proteobacteria* → α -*Proteobacteria* → β -*Proteobacteria* → γ -*Proteobacteria* (Gupta, 1998a, 2000a, b).

The identified signature sequences in various proteins are also useful for deterministic purposes and based

upon the presence or absence of these signatures, it is now possible to place with a high degree of confidence any given bacterial species within one of the main groups that could thus far be distinguished (Gupta, 1998a, 2000a, b). In the present study, we have used the signature sequence approach to understand the phylogenetic placement of *F. succinogenes* within the domain *Bacteria*. For this purpose, we have amplified by PCR and sequenced segments of the Hsp60 (*groEL*), Hsp70 (*dnaK*), CTP synthase and alanyl-tRNA (Ala-tRNA) synthetase genes from *F. succinogenes*, which are known to contain useful signature sequences that are distinctive for different bacterial divisions (Gupta, 1998a, 2000a). The analyses of these signature sequences provide strong evidence that *F. succinogenes* shows a specific relationship to the *Chlamydia* and CFB groups of species. These signature sequences also provide evidence that this group of bacteria evolved after the branching of Gram-positive bacteria, *Deinococcus-Thermus* groups, green non-sulphur bacteria, cyanobacteria and spirochaetes, but prior to the branching of *Proteobacteria* (Gupta, 1998a, 2000a).

METHODS

PCR amplification and sequencing. Purified DNA of *F. succinogenes* S85 (type strain; ATCC 19169) was generously made available to us by Dr C. Forsberg (Guelph University, Ontario, Canada) and Dr J. Yanke (Agriculture Canada Research Centre, Lethbridge, Alberta). Degenerate oligonucleotide primers, in opposite orientations, were designed for highly conserved regions in the indicated proteins that flanked the previously identified signature sequences. The regions of high sequence similarity suitable for primer design were identified by carrying out multiple sequence alignments of homologues for these proteins from different species. These PCR primers, because they correspond to highly conserved regions, can be used to amplify homologous gene fragments from different species. All primers were synthesized at MOBIX (The Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University).

Hsp70 gene. The PCR amplification of the Hsp70 gene fragment was carried out in two segments. Initially, a fragment of 1080 bp was amplified using degenerate primers for two highly conserved regions in the Hsp70 protein (Galley *et al.*, 1992). The forward primer was for the amino acid sequence GIDLGT (nucleotide sequence 5'-GGNATHGAYYTNGG-NACNAC-3') and the reverse primer for the sequence NPDEAVA (nucleotide sequence, 5'-GCNACNGCYTCRT-CNGGRTT-3', where R represents A or G; Y represents C or T; H represents T, A, or C; and N represents A, G, C, or T). After this fragment was cloned and sequenced, the sequence was extended in the direction of the 3'-end by designing a specific forward primer near the 3'-end of the sequence (5'-AGGAACCGAACAAGACTG-3') and a degenerate primer in the reverse orientation for the conserved sequence QIEVTFD towards the C-terminal end of the protein (nucleotide sequence 5'-TCRAANGTNACYTCDATYTG-3') (Galley *et al.*, 1992). This led to the amplification of a 361 bp fragment whose sequence was combined with the original Hsp70 fragment to obtain a contiguous sequence of 1400 bp. The nucleotide and corresponding protein sequence were deposited in GenBank under the accession number AY017382.

Hsp60 gene. The amplification of the Hsp60 gene fragment

was carried out using a degenerate forward primer for the amino acid sequence GPKGRN (5'-GGNCCNAARGGN-A(C)GNAAYGT-3') and a degenerate reverse primer for the sequence AVKAPGFGE (5'-TCNCCRAANCCNGGNGCY-TTNACNGC-3') (Rusanganwa *et al.*, 1992). These primers amplified a 756 bp fragment of the Hsp60 protein. Sequence data were deposited in GenBank under the accession number AY017380.

Alanyl-tRNA synthetase. A 171 bp fragment of Ala-tRNA synthetase was amplified using a degenerate forward primer for the amino acid sequence FTNAGM (5'-TTYACNAAY-GCNGGNATG-3') and a reverse primer for the sequence HTFFEM (5'-CATYTCRAARAANGTRTG-3'). Protein and nucleotide sequences were deposited in the GenBank database under the accession number AY017381.

CTP synthase. A 401 bp fragment of CTP synthase was amplified using a degenerate forward primer for GICLGMQ (5'-GGNATHTGYYTNGGNATGCA-3') and a reverse primer for QFHPEF (5'-AAYTCNGGRTGRAAYTG-3'). Sequences for this gene and its corresponding protein were deposited in GenBank under the accession number AY017383.

PCR. PCR reactions, of 10 µl volumes (approx. 0.2 µg DNA per reaction), were optimized for Mg²⁺ concentration (in the range of 1.5–4 mM) for each set of primers. PCR was carried out using a Techne Progene thermocycler, over 30 cycles (15 s at 94 °C, 15 s at 55 °C, 1 min at 72 °C) with an initial 1 min hot start at 94 °C, and a final extension step (15 s at 94 °C, 15 s at 55 °C, 7 min at 72 °C). The DNA fragments of the expected size were purified from 0.8% (w/v) agarose gels (using a GENECLEAN kit), and subcloned into the plasmid pCR TOPO 2.1 using a TA cloning kit (Invitrogen). After transformation of *Escherichia coli* JM109 cells with the plasmids, the inserts from a number of positive clones were sequenced. The fragments of less than 0.6 kb in length were sequenced by the dideoxy chain termination method using a T7 sequencing kit (Pharmacia). The larger fragments were sequenced by MOBIX. Sequences of all cloned fragments were run through a BLAST search to ensure the DNA was from a novel source.

Signature sequence analysis. The nucleotide sequences for the four *F. succinogenes* genes were translated and added to multiple alignments of homologous proteins (sequences from various known bacteria acquired from BLAST searches, www.ncbi.nlm.nih.gov). Alignments were carried out with either ALIGN or CLUSTAL W software.

RESULTS

Signature sequences and their usefulness in deducing evolutionary relationships

Signature sequences in proteins, defined as conserved inserts or deletions (i.e. indels) that are restricted to specific taxa provide a powerful means to deduce the evolutionary relationship among distantly related organisms (Gupta, 1998a, b). In phylogenetic trees based on most gene sequences, including that of the 16S rRNA, evolutionary relationships between the major taxa within each domain are generally not resolved; this has been recognized as a major weakness or limitation of the tree construction approach (Olsen & Woese, 1993; Ludwig & Schleifer, 1999; Gupta, 2000c). The inability of the phylogenetic trees to resolve such relationships is in large part due to the dependence of the derived inference upon a number of different variables and

assumptions (e.g. reliability of the sequence alignment, regions of sequences that are retained or excluded in phylogenetic analysis, number and range of species included, differences in the evolutionary rates, base compositional differences, phylogenetic methods employed, etc.) (Gupta, 1998b). Hence, there is a clear need for alternative approaches that are minimally affected by the above variables and assumptions, and which are capable of resolving such relationships. The presence of shared signature sequences in proteins provides such an approach.

The use of the signature sequence approach for understanding the evolutionary relationship is based on the rationale that when a conserved indel of defined length and sequence, and flanked by conserved regions to ensure that the observed changes are not due to incorrect alignment or sequencing errors, is found at the same position in homologues from different species, then the simplest and most parsimonious explanation for this observation is that the indel was introduced only once during the course of evolution and then passed on to all descendants. A well-defined indel in a gene or protein thus provides a very useful milestone for evolutionary events, since all species emerging from the ancestral cell in which the indel was first introduced are expected to contain the indel, whereas all species that existed before this event or which did not evolve from this ancestor will lack the indel (Gupta, 1998a, c). To interpret whether a given indel in a particular protein is the result of an insertion or deletion event, and its possible implications regarding the relative branching order of different groups, a reference point is essential. Such a reference point is provided by the root of the prokaryotic tree. Several lines of evidence show that the root of the prokaryotic tree lies between the Archaea and Gram-positive bacteria (Iwabe *et al.*, 1989; Brown & Doolittle, 1995; Gupta, 1998a, b, 2000b), both of which are prokaryotic organisms bounded by a single membrane. Based on this rooting, the signature sequences in various proteins can be clearly interpreted and the branching order of different groups from the common ancestor can be logically deduced as described in earlier work (Gupta, 1998a, 2000a).

Deducing the phylogenetic placement of *F. succinogenes*

To employ the signature sequence approach for determining the phylogenetic placement of any given species, one begins by successively examining the signature sequences in different proteins that exclude the placement of the species in certain groups and at the same time show specific affinity of the species, or narrow down its placement, in certain other group(s) (Gupta, 1998a). One prominent signature consists of an indel of 21–23 aa, present in the N-terminal quadrant of Hsp70 protein, which distinguishes all true Gram-negative bacteria (defined by the presence of an inner and an outer membrane) from Gram-positive bacteria (Gupta, 1998a, 2000a). In earlier work, we have presented evidence that this indel represents an insert in the Gram-

Fig. 1. Partial alignment of Hsp70 sequences showing a signature sequence consisting of an insert of 21–23 aa (box 1), which is specific for Gram-negative (or diderm) bacteria. The presence of this insert in *F. succinogenes* provides evidence that it is a Gram-negative bacterium. A smaller 2 aa insert (box 2) within the large insert is distinctive of the proteobacterial species. This insert is absent in *F. succinogenes*. Dashes indicate an identical amino acid to that found in the *E. coli* sequence shown on the top line. Accession numbers of the sequences are shown in the second column. The asterisks (*) denote sequences identified by BLAST search from the NCBI Unfinished microbial genome database (www.ncbi.nlm.nih.gov/BLAST/unfinishedgenome/html). Abbreviations used are as follows: *A.*, *Agrobacterium*; *Aph.*, *Aphanotoce*; *Aqu.*, *Aquifex*; *Bac.*, *Bacillus*; *Bor.*, *Borrelia*; *Bord.*, *Bordetella*; *Burk.*, *Burkholderia*; *C.*, *Caulobacter*; *Cb.*, *Chlorobium*; *Chl.*, *Chlamydia*; *Cf.*, *Chloroflexus*; *Clo.*, *Clostridium*; *Cyto.*, *Cytophaga*; *D.*, *Deinococcus*; *Desulf.*, *Desulfovibrio*; *E.*, *Escherichia*; *Eh.*, *Ehrlichia*; *Ery.*, *Erysipelothrix*; *F.*, *Francisella*; *Fi.*, *Fibrobacter*; *Fl.*, *Flavobacterium*; *Geo.*, *Geobacter*; *H.*, *Haemophilus*; *He.*, *Helicobacterium*; *Hel.*, *Helicobacter*; *L.*, *Lactococcus*; *Le.*, *Legionella*; *M.*, *Mycoplasma*; *Meg.*, *Megasphaera*; *Myc.*, *Mycobacterium*; *Myxo.*, *Myxococcus*; *Nei.*, *Neisseria*; *Nit.*, *Nitrosomonas*; *Pas.*, *Pasteurella*; *Po.*, *Porphyromonas*; *Poly.*, *Polynucleobacter*; *Pse.*, *Pseudomonas*; *R.*, *Rhizobium*; *Rho.*, *Rhodobacter*; *Sta.*, *Staphylococcus*; *Str.*, *Streptomyces*; *Strep.*, *Streptococcus*; *Sy.*, *Synechocystis*; *Syn.*, *Synechococcus*; *T.*, *Thermotoga*; *The.*, *Thermomicrobium*; *Ther.*, *Thermus*; *Tre.*, *Treponema*; *Xy.*, *Xyllella*.

negative bacteria rather than a deletion in the Gram-positive bacteria (Gupta & Singh, 1992; Gupta & Golding, 1993; Gupta, 1998a), indicating the ancestral

nature of the Gram-positive group. Hsp70 is a highly conserved protein, which carries out an essential chaperone function in the cell (Lindquist & Craig, 1988).

			144	178
	E.coli	P06139	IAQVGTISA	N SDETVGKLI AEAMDKVGKEGVITVE
	H. ducreyi	P31294	-E-----	-----Q-E-----
	Cox. burnetii	P19421	-----	---KSI-DI-----E-----
	Buch. aphidicola	Q59177	T-----	A-K-A-----E-ND-----
	Enter. aerogenes	d1026143	-----	-----
	Er. aphidicola	d1026167	-----	---A---E---Q-E-----
	K. ornithinolytica	BAA25231	-----	-----
	Ser. ficaria	BAA25221	-----	-----E-----
	Thio. ferrooxidans	P29134	V-----	---DSI---I-----E---N-----
	Xy. fastidiosa	AE003849	---A---	---SI-NI-----K-----I-
	Yer. enterocolitica	d1003671	-----	---S---E---Q-E-----
	Vib. parahaemolyticus	AAF27528	-----	---AS-NI-----ER-RD-----
	Bord. bronchiseptica	*	---S---	---ASI-QI---D-----
	Nei. flavescens	CAA80531	---S---	---Q-AI-----E-----
	Par. denitrificans	BAA36516	V-----	GESFI-QQ-----QR--N-----
	A. tumefaciens	P30779	V-----	GERQI-LD-----QR--N-----
	Brad. japonicum	P35861	-----	G-AEI--F-SD--K--N-----
	Ri. tsutsugamushi	P16625	---A-V-S	G-REI-EK--N--KQ-Q-----
	Eh. chaffeensis	L10917	---A---	G-KNI-SK--QCVQE--D-----
	Bar. bacilliformis	P35635	---A---	GA-DI--M--D--E--N-----
	Brucella abortus	P25967	V-----	AEAEI--M-----Q--N-----
	Eh. canis	2290612	---A---	G-KNI-VK--QCVQE--D-----
	Rho. capsulatus	P95678	V-----	GEAQI-RF--D-SQ--N-----
	Camp. jejuni	e1287421	---A---	---KI-N--D--E--D-----
	Hel. pylori	P42383	-T-A---	---HNI-----D-E--D-----
	Law. intracellularis	AAC36500	-----	---T-I-NI-----A--G-----
	Geo. sulfurreducens	*	-----	N-K-I-DI--Q-E-----
	Desulf. vulgaris	*	-----	---S-I-NI-----S-----
	Chl. muridarum	AAF39243	---A---	N-AEI-N-----E--N-S-----
	Chl. caviae	CAA35766	---A---	N-AEI-N-----E--N-S-----
	Cb. tepidum	*	-----	N-PEI-E-----D-----
	Fi. succinogenes	AY017380	---A---	N-PEI-E-L-N--E--ND-----I-
	Cyto. aquatilis	AF335327	-K-IAS--	N--VI-E--T-FA-----
	Bact. forsythus	CAB43992	-EH-AK--	G--GI-----Q-K-----
	Rhodo. marinus	AAD37976	---A---	G-KAI-Q--D-FE--D-----
	Po. gingivalis	D17398	-EH-AK--	G--NI-S--R--K-----
	Fl. ferrugineum	AF335328	-Q--A---	N--I-----FA-----
	Aqu. aeolicus	2984379	-E--A---	N-PEI--I--D--EE--D-----
	Bor. burgodferi	P27575	---AS---	N-SYI-EK-----D-----
	Tre. denticola	*	V-H-ASV--	N-AEI--I--D-IA--D--D-G
	Lep. interrogans	L14682	---N-AS---	N-N-I-N--D-----D-----
	Syn. vulcanus	d1024722	---AA---	G N--E--RM--D-----SL-
	Sy. sp. PCC6803	2506274	---A---	G N--E--QM--N--Q--SL-
	Syn.sp. PCC6301	48022	---A---	G N-FE--QM--D-----K--SL-
	Syn. sp. PCC7942	P22879	---A---	G N-FE--QM--D-----SL-
	Ther. aquaticus	P45746	-EE-A---	N-PE-----D--E-----I---
	D. proteolyticus	AF130448	-KK-AG--	N-PQ--EE--S-----I---
	D. radiodurans	AAF10186	-KK-AG--	N----QE--S-----I---
	Myc. leprae	P09239	--ATAA--	G-QSI-D-----N-----
	Myc. tuberculosis	P06806	--ATAA--	G-QSI-D-----N-----
	T. maritima	unpubl.	--H-AA--	NSAEI-E-----ED-----
	Str. albus	Q00767	--A-AAL--	Q-KQ-E-----D-----
	Strep. pyogenes	e187584	---AAV-S	RS-K--EY-S--ER--ND-----I-
	Clo. perfringens	P26821	--R-AA--	A--KI-----D--E--N-----
	Clo. difficile	AAF80372	-S--AS--	G--E-----EI--D-----
	L. lactis	X71132	---A-V-S	RS-K--EY-SD--ER--SD-----I-
	Ent. faecalis	*	---AAV-S	GS-K--Q--D--E--ND-----I-
	Sta. intermedium	2554919	---A---	A--E--RY-S--E--ND--SI-
	Lac. johnsonii	AAF75593	---ASV-S	ASTE--N--RD--E--HD--I-
	Bac. anthracis	*	---AA---	A--E--Q-----ER--ND--L-
	M. pneumoniae	P78012	-E--AA--S	GSKEI-----Q--AL--N--TD

Fig. 2. Excerpts from the Hsp60 sequence alignment showing a signature sequence that distinguishes Gram-positive bacteria, *Deinococcus-Thermus* and green non-sulphur bacteria from the other groups of bacteria. The presence of this insert in *F. succinogenes* provides evidence that it belongs to the latter group. Dashes denote identity to the amino acid in the top line. Additional abbreviations in the species names are as follows: *Bact.*, *Bacteroides*; *Bar.*, *Bartonella*; *Brad.*, *Bradyrhizobium*; *Buch.*, *Buchnera*; *Camp.*, *Campylobacter*; *Cox.*, *Coxiella*; *Ent.*, *Enterococcus*; *Enter.*, *Enterobacter*; *Er.*, *Erwinia*; *K.*, *Klebsiella*; *Lac.*, *Lactobacillus*; *Law.*, *Lawsonia*; *Lep.*, *Leptospira*; *Par.*, *Paracoccus*; *Rhodo.*, *Rhodothermus*; *Ri.*, *Rickettsia*; *Ser.*, *Serratia*; *Thio.*, *Thiobacillus*; *Vib.*, *Vibrio*; *Yer.*, *Yersinia*.

Fig. 3. Signature sequence in the alanyl-tRNA synthetase gene, which is present only in the *Proteobacteria* and the *Chlamydia* and CFB groups of species. The presence of this insert in *F. succinogenes* indicates a relationship to these taxa. Dashed lines indicate an identical amino acid match to that of *E. coli*, given on the top line. Additional abbreviations are: *Sal.*, *Salmonella*; *Shew.*, *Shewanella*; *Spiro.*, *Spiroplasma*; *Ur.*, *Ureaplasma*; *Z.*, *Zymomonas*.

Within Gram-negative bacteria two of the deepest branching divisions, *Deinococcus-Thermus* and green non-sulphur bacteria, can be distinguished from the others based on a signature present in the Hsp60 (GroEL) protein (Gupta, 1998a). This signature consists of a 1 aa insert that is commonly shared by species from various other groups of Gram-negative bacteria, including different groups of *Proteobacteria*, the *Chlamydia* and CFB groups, spirochaetes and cyanobacteria, but is not present in green non-sulphur bacteria and *Deinococcus-Thermus* (Gupta, 1998a). This insert, as expected, is also not found in any Gram-positive bacteria. Similar to Hsp70 protein, the Hsp60 protein is also a highly conserved protein found in all bacteria (Gupta, 1995), and the indicated signature is highly

		377		442
	H. influenzae	1172792	GICLGMQIALIEARNVAGLTKANSS FDK	DCEQPVVALITE WQDAEGNTEV RTDESGLGGTMRIG
	E. coli	147478	-----V--D---H--NMEN---T--VP	--KY-----R-EN--V--SEK-----
	Nit. europaea	3493582	-----L-V--FS--RLQ-EN-H-T--P	-TPY--LG---R-RC-RV-KSAQT-----
	Xy. fastidiosa	AAF84097	---Y---A-VVD---H---EG---T--N-R	QSPH--I----RTTT-EV-RDEK-----
	Le. pneumophila	*	-----T-V--F---V---G---T--N-	ETLY--LG--S--M--D-SKQI--DENT-----
	Pas. multocida PM70	*	-----V-Y---D---D---T--R-R	T-DY---G---I-T-----A-----
	Thio. ferrooxidans	*	-----L-VV-F--HR--MN---T--L-P	QTPA--IT-M--S-P--HKAYEEGNG-----
	K. pneumoniae	*	-----V---F---MEN---T--VP	--KY-----R-ED--V--SEK-----A
	Yer. pestis	*	-----V--M-F---MEN---T--VP	--KY-----R-ED--V-I--E-----V-
	Sal. typhi	*	-----V---F---MDN---T--VP	--KY-----R-ED--V--SEK-----I----
	Shew. putrefaciens	*	-----V---F---MAD-H-T--N-	ATPF--G---V-E--V-QHEA-----
	Pse. aeruginosa	AAG07025	-----V-V---L-WSD---T---	SSGH--G---T-A--I--EA-----
	Vib. cholerae	AAF95590	-----V---M-F---MEG-H-T--N-	NTKY--G---V-G--V-ESEK-----
	Nei. meningitidis	CAB84970	-----D---D---KG---T--L	K-AA---D--T-D-SV-TDESA-----
	Bord. bronchiseptica	*	-----L-V--F--H---GG---T--P	AAPH-----M-R--RV-RDNS-----K-
	C. crescentus	*	---F---M-V--TL---IKD-S---G	PTER---GIM--IKGNE-VQ--RAND-----
	Desulf. vulgaris	*	-----C-V--F---M--ED---E-FNEL	SKNKVIYLMTEWFDHRRQAV-R--DSS--K-----
	Hel. pylori	3183132	-----L-IV-FC---L--KG---T--NQ	R--Y---Y--GD--FM-QNHQKQ--YN-P-----
	Camp. jejuni	CAB72520	-----L--V-F---LK-KDV---NE	K-QN---Y--D--FM-TN-EKQI--AKTP-----
	Cb. tepidum	*	-----C-T--F---ICD-PD---T--N-	RTRF--ID-MEH--QKKVKEK-----
	Fi. succinogenes	AY017383	-----MLA--F--D-L-WKD---T--E	NTTH--ID-MD--QKNVTEK-----
	Aqu. aeolicus	2983754	-----LMA--F---L-FSN---T--P	-TPF--IDIME--QKKVOK-----
	Chl. trachomatis	2494759	-----ALVV---LDKPL---M-MNP	ETPD---CMMEG--QDSVVK-----
	Chl. pneumoniae	4376510	-----VLVV---LN-DQ---L-M-P	NTPH--I-YVMEG--QDPLVAT-----
	Chl. muridarum	AAF39308	-----ALVV---YALS-PL---L-M-P	NTPD---CMMEG--Q-TMIK-----
	Chl. psittaci	*	-----VLVV---MD-E---T-M-Q	-TPH--ICMMDG--QAALVAT-----
	Bor. burgdorferi	2688502	-----L-L-V--F---C-ILD-DTE-NLARDKPLKS	-IH-LP--KQGIK-K-A-----
	Tre. pallidum	AE001210	-----V--F---LL-AS-H-R--AV	-TPH--D-LPG--CV--TPT--SL--
	Tre. denticola	*	-----SA---L-ALHIEE-----	NAKN--ID-MPD--QK-DVKI--L--
	Po. gingivalis	*	-----CMV-----L-FKD--TT-IES	NI-HK-ID-MD--QKTVT-M--S--
	Syn. sp. PCC7942	2494760	-L---A-V-DW-----DG---A--P	ETPH--I--LP--QQ-VV-----
	Sy. sp. PCC6803	2494761	-L---CSV--W---K-PE---A-ET	ETPN--IN-LP--QQ-VV-----
	D. radiodurans	AE002001	-----V---H---IED---A--E	YAKNK-ID-MP--QLEVAGM-----
	Myc. bovis	1850588	-L---L-CIV--A--S---N---A--P	-TPD--I-TMPD--QK-IE-----L--
	Myc. leprae	1709961	-L---L-CIV--AT-S---VQ---A-EP	ATPD---ISTMAD--QK-VE-----L--
	Myc. tuberculosis	2326742	-L---L-CIV--A--S---N---A--P	-TPD--I-TMPD--QK-VE-----L--
	Myc. avium	*	-L---L-CIV--A--S---AG---A--P	-TPD--ISTMAD--QENIEN-----L--
	Str. coelicolor	5738492	-L---L-CIVV--A--L--VAD---T--P	ATAH---STMA--QI-IE-M---L--
	Sta. aureus	*	-----L-TV-FS---L--EG-H-A-L-P	ATPY-IID-LP--QK-IE-----L--
	Bac. subtilis	131733	-----V-S-----L--KG-H-A-I-P	STQY-IID-LP--QK-VE-----L--
	Bac. anthracis	*	-----L-S--F---L--EG---INP	-TPYAIID-LP--QK-VE-----L--
	Bac. stearothermophilus	*	-----L-S--F--H-V--SG-H---P	NTPH-IID-LP--QK-VE-----L--
	Ent. faecalis	*	-----M-CV-FG---V--ED-G-A-TNP	-VTNNIID-MAD--QENIEN-----L--
	Strep. pneumoniae	*	-V---LTCV--F--H-L--EG---A-LAP	ETKY-IIDIMRD--QI-IE-M---L--
	Strep. pyogenes	*	-----LTCV--F--H-LNMEG---F-LEP	STKY-IIDIMRD--QI-IE-M---L--
	Strep. mutans	*	-----LTCV---H-LN-EG---A-L-P	-TKY--IIDIMRD--QI-IE-M---L--
	L. lactis	3757664	-----LTAV--F---L--EG-H-FA-L-P	ETKY--IIDIMRD--QV-VE-M---
	M. capricolum	629288	-----T-SI--DLLNW-D-D-T--N-	NTTH-IFDY-K--GI-RDNI--L--
	Clo. acetobutyl.	*	-----C-V--F---L--EG-H---I-P	QTKY--ID-MPD--QK-ID-K-----
	T. maritima	AE001748	-----LMV--F---F-YKE---T--P	NTPY---D-ME--QKRILK-----

Fig. 4. A signature sequence consisting of a 10 aa insert in CTP synthase which is distinctive of the proteobacterial species. The absence of this insert in *F. succinogenes* provides evidence that it does not belong to the proteobacterial group.

reliable, with no exceptions observed in more than 200 sequences available from different bacterial species (Gupta, 2000a; unpublished results). To examine the presence of this signature in *F. succinogenes*, a 756 bp fragment of the Hsp60 gene was amplified by PCR using degenerate primers based on highly conserved regions in the Hsp60 protein. The sequence alignment of a portion of this region from *F. succinogenes* with other bacterial species is presented in Fig. 2. As seen, the Hsp60 from *F. succinogenes* contains the indicated signature, providing evidence that this species does not belong to the green non-sulphur bacteria or the *Deinococcus-Thermus* groups.

We next examined the presence in *F. succinogenes* of a signature sequence found in the Ala-tRNA synthetase gene. The signature in this protein consists of a 4 aa insert that is uniquely found only in the homologues from various *Proteobacteria* and the *Chlamydia* and CFB groups of species (Gupta, 2000a). Ala-tRNA

synthetase, which carries out an essential role in protein synthesis, is also found in all completed prokaryotic genomes (Gupta, 2000a). Using degenerate primers for highly conserved regions in this protein which flank this sequence signature, we successfully amplified a 171 bp fragment that corresponded to the Ala-tRNA synthetase gene. The alignment of the translated sequence with other species shows that this insert is present in the Ala-tRNA synthetase from *F. succinogenes* (Fig. 3). In view of the specificity of this sequence signature, the presence of this insert in *F. succinogenes* provides strong evidence that this species belongs to one of these two groups of bacteria.

A number of signatures have been identified in proteins that are specific for the proteobacterial division and serve to distinguish members of this group from other divisions of bacteria. The protein CTP synthase contains a 10 aa insert that is uniquely shared by all proteobacterial species, but is not found in the *Chlamydia* and

		190	233
		IAVYDLGGTDFDISIIIEI	GEKTFEVLATNGDTHLGGEDFD
Proteobacteria	<i>E. coli</i>	SP/P04475	DEVD
	<i>Sal. typhi</i>	*	-----E-----
	<i>Vib. proteolyticus</i>	AAF27648	A-----Q-----F-----
	<i>Xy. fastidiosa</i>	G82570	A-ME--HQ---S---F---G---
	<i>Thio. ferrooxidans</i>	*	-----K-----
	<i>Buchnera. sp.</i>	2351216	-----NF--Q---R-----
	<i>Pas. haemolytica</i>	2738813	A---HQ---F-----
	<i>Cox. burnetii</i>	3688231	AD---QQ-----F-----
	<i>Psychrobacter sp. St1</i>	AAF70337	A---HQ-----F-----
	<i>Le. pneumophila</i>	3122002	-----NF--Q---G-N---
γ and β	<i>H. influenzae</i>	1542849	-----SN--DQ-----
	<i>Shew. putrefaciens</i>	*	SD---DNQI---S---F-----
	<i>F. tularensis</i>	1352284	V-----V-----A---HQ-----F-----
	<i>Pse. syringae</i>	AAD31868	VI-----V-V---ANL--D-Q-----F-----
	<i>Nei. gonorrhoeae</i>	*	AD---MQ---S---F-----
	<i>Bord. pertussis</i>	*	AD---MQ---S---F-----
	<i>Burk. cepacia</i>	1169373	A-E---HQ-----F-----
	<i>Nit. europaea</i>	2094746	-----VL---DGV---KS---F-----
	<i>R. meliloti</i>	GB/L36602	-----V-L---SDGV---KS---F-----
	<i>Ri. prowazekii</i>	3860750	-----V-VL---DGV---KS---F-----
α	<i>B. ovis</i>	1027504	-----V-L---DGV---KS---F-----
	<i>C. crescentus</i>	GB/M95799	-----F-----V-L-L-DGV---K-----
	<i>Eh. sennetsu</i>	*	-----V-L-L-DGV---KS---F-----
	<i>Rhodopseud. sp.</i>	2058265	-----L---DGV---KS---F-----
	<i>Brad. japonicum</i>	1769954	-----VL---DGV---KS---F-----
	<i>A. tumefaciens</i>	SP/P20442	-----VL---DDGL---KS---F-----
	<i>Rho. capsulatus</i>	1373328	-----L-L-NAGV---KS---F-----
	<i>Myxo. xanthus</i>	1805284	-----L-L-DGV---KS---F-----
	<i>Geo. sulfurreducens</i>	*	-----L-V-DNVV---R-----F-----
	<i>Desulf. vulgaris</i>	*	-V-----VTVL-T-DNVV---G-NAF---D---
δ and ϵ	<i>Camp. jejuni</i>	CAB73024	-M-----VTVL-T-DNVV---G-AF---D---
	<i>Hel. pylori</i>	2072520	GG-----Y---LD-S-GV-Q-----Y---TPLVH--
	<i>The. roseum</i>	1813674	-----F-----VL-L-DGV---KS---D---
	<i>Fl. ferrugineum</i>	AF013111	V-----L---DGM-SAK---M---DN--
	<i>Fi. succinogenes</i>	AY017382	-I-----VL-L-DGV---S-----D---
	<i>Cyto. aquatilis</i>	AF013110	-----F-----L---DGV---S-----D---
	<i>Chl. psittaci</i>	*	-----L-L-DGV---KS---D---
	<i>Tre. pallidum</i>	3322484	-----L-L-DGV---KS---D---
	<i>Po. gingivalis</i>	4001713	-L---F-----V-L-G-DGVI---K-A-----ANI-
	<i>Aqu. pyrophilus</i>	3377943	-L---F-----GVS-LE-GVI---KV-A-----ANI-
Other Gram (-)ve Bacteria	<i>Aqu. aeolicus</i>	AE000717	-----LDV-ADGV---S-S-----D-W-
	<i>Planctomyces maris</i>	2645004	-----Y---LEL-GDGV---K-----DN--
	<i>Rhodo. marinus</i>	*	V---F-----L-L-GGV---KS-D-----D---
	<i>Cb. tepidum</i>	AF130447	V-----L-L-DGV---KS-----DN--
	<i>Bor. burgdorferi</i>	SP/P28608	-L-F-----V-L-V-DGV---K-S---Q---N---
	<i>Anab. variabilis</i>	2073390	-L-F-----V-VLKV-NGV---K-S---Q---N---
	<i>Syn. sp. PCC7942</i>	GB/D29668	-L-F-----V-L-V-GV-----S-----D---
	<i>Sy. sp. PCC6803</i>	SP/P22358	-L-F-----V-L-L-DGV---KS-S---S-A---
	<i>D. proteolyticus</i>	1813672	VL-F-----V-L-L-DGV---KS-A-----A---
	<i>D. radiodurans</i>	AE001875	VL-F-----V-L-L-GV---K-S---S-M-
Gram (+) ve Bacteria	<i>Ther. aquaticus</i>	GB/Y07826	-L-----V-VL-L-DGV---KS---N---D---
	<i>Clo. acetobutyl.</i>	232001	VL-----V-VL-L-DGV-Q-S---R---D---
	<i>Lac. sakei</i>	3688419	VL-----V-L---GVI---I---A-NN---D---
	<i>T. maritima</i>	3319784	-L-F-----V-L-L-DGVV---K---N---D-W-
	<i>Str. griseus</i>	3023655	VL-----V-L-L-DGV---R-A-NR---D---
	<i>Bac. megaterium</i>	118721	-L-----V-L-L-ADG---IS-A-NR---D---
	<i>Bac. anthracis</i>	*	-L-F-----V-L-L-ADG---S-S-N---D---
	<i>Ur. urealyticum</i>	*	-L-F-----V-L-L-DGV-D---A-NK---D---
	<i>Strep. agalactiae</i>	3122025	-L-F-----V-L-L-GVV---R-S-S-N---D-W-
	<i>Myc. avium</i>	*	-L-F-----V-L-L-DGVV---R-A-NE---D-W-
Gram (+) ve Bacteria	<i>Cor. diphtheriae</i>	*	VL-F-----V-L-L-DGV---S-A-NK---D---
	<i>Sta. aureus</i>	1169381	VL-F-----V-L-L-ADG---S-A-NK---D---
	<i>Ery. rhusiopathiae</i>	1169374	-L-F-----V-L-L-DGVV---K---NN---D-W-
	<i>Str. coelicolor</i>	461946	-L-F-----V-L-L-DGV-D---A-NNK---D---
	<i>L. lactis</i>	1169377	-L-F-----V-L-L-DGV-D---A-NK---DN--
	<i>Strep. mutans</i>	2495353	-L-----V-L-L-GGS-D-I-S-NNK---DN--
	<i>M. capricolum</i>	530439	-L-F-----V-L-L-DGV-D-S-A-NN---D---
	<i>Ent. faecalis</i>	*	-----

Fig. 5. A 4 aa insert in Hsp70 that is specific for the β and γ -subdivisions of *Proteobacteria*. This insert is also absent in *F. succinogenes*, supporting the inference from CTP synthase and Hsp70 signatures that this species is not a part of the proteobacterial group. Additional abbreviations are: *Anab.*, *Anabaena*; *B.*, *Brucella*; *Cor.*, *Corynebacterium*, *Rhodopseud.*, *Rhodopseudomonas*.

CFB groups, or other divisions of bacteria (Gupta, 2000a). Using degenerate primers for highly conserved regions in CTP synthase, we were able to amplify a 401 bp fragment for the CTP synthase gene from *F. succinogenes* which contained this sequence region. The

alignment of the translated sequence from *F. succinogenes* with other bacterial species is presented in Fig. 4. From the alignment it is clear that the 10 aa insert common to proteobacterial species is not present in *Fibrobacter*. In addition to CTP synthase, Hsp70 also

contains a 2 aa insert within the large insert that is distinctive of the proteobacterial species (box 2 in Fig. 1). This 2 aa insert is not found in the *F. succinogenes* Hsp70 either, providing further evidence that *Fibrobacter* does not belong to the proteobacterial group. The Hsp70 protein contains yet another signature consisting of a 4 aa insert that is specific only for the β and γ -subdivisions of *Proteobacteria* (Fig. 5) (Gupta, 2000a). As expected, the Hsp70 homologue from *F. succinogenes* does not contain this insert, providing additional evidence that *Fibrobacter* does not belong to these groups of *Proteobacteria*. Based on the signature sequences in different proteins, it is thus clear that *Fibrobacter* is specifically related to the *Chlamydia* and CFB groups of species.

DISCUSSION

In the past 15–20 years, phylogenetic analyses based on 16S rRNA sequences have become the primary means for understanding the evolutionary relationships among prokaryotes. Based on the branching positions of species in the rRNA trees, a number of major groups or divisions among bacteria have been identified (Woese, 1987; Olsen & Woese, 1993; Ludwig & Schleifer, 1999). The analysis based on 16S rRNA sequences has proven invaluable and generally quite successful in the assignment of various bacterial species into one of these groups (Maidak *et al.*, 1999). However, one major limitation of the phylogenetic trees based on 16S rRNA (or those based on different protein sequences) is that they are unable to resolve reliably the branching orders of higher taxa or main groups within bacteria (Gupta, 1998a; Ludwig & Schleifer, 1999; Olsen & Woese, 1993). Due to this limitation, a number of important issues that are central to understanding bacterial phylogeny still remain unresolved. Such questions include: (i) how the major divisions within the *Bacteria* are related to each other, and (ii) in what order these groups branched off from the common ancestor (Gupta, 2000b, c). The inability of the rRNA trees (or those of various proteins) to resolve these relationships has led to growing acceptance of the notion that these problems are basically unsolvable and that all of the main groups within the *Bacteria* probably branched off directly from the common ancestor (Doolittle, 1999; Ludwig & Schleifer, 1999; Woese, 1998).

In this context, the signature sequence approach developed in our recent work provides a new and powerful method for examining the evolutionary relationships among the species (Gupta, 1998a, 2000a). In contrast to the phylogenetic trees, where the derived inference is based upon a large number of variables (e.g. reliability of the alignment, regions of sequences that are retained or excluded, number and range of species included, differences in evolutionary rates, base compositional differences, phylogenetic methods employed, etc.) which contributes to the uncertainties of the end results (Gupta, 1998a), the evolutionary inference in this new approach is based entirely on the presence or absence of highly

conserved sequence features. These sequence characteristics comprise indels of defined lengths present in highly conserved parts of the molecules, which makes interpretation of the data generally quite simple, unambiguous and based on minimal assumptions (Gupta, 1998a). Based upon a large number of conserved indels in various proteins that have been introduced at various stages in evolution, it is now possible to clearly define most of the major groups within the *Bacteria* and to discern logically their branching order from the common ancestor (Gupta, 1998a, 2000a, b). These signatures also permit the placement of any bacterial species into one of these groups based on the presence or absence of specific signatures in different proteins (Gupta *et al.*, 1999; Gupta, 2000a).

In the present work, the signature sequence approach was used to determine the phylogenetic placement of *Fibrobacter succinogenes* within the domain *Bacteria*. Results presented here show that *F. succinogenes* is most closely related to the *Chlamydia* and CFB groups of species. A summary of the signature sequences that were used to derive this inference is presented in Fig. 6. The presence of the large 21 aa insert in *F. succinogenes* Hsp70, which is distinctive of all Gram-negative bacteria and not found in any Gram-positive bacteria, places *Fibrobacter* in the Gram-negative group. This indel has previously been shown to be due to an insertion in the common ancestor of Gram-negative bacteria, rather than a deletion in the Gram-positive group. This makes the Gram-positive bacteria an ancestral lineage and it provides a reference point for interpreting other signature sequences. The placement of *Fibrobacter* in the Gram-negative group is also supported by the signature in the Hsp60 protein, which additionally also excludes *Fibrobacter* from the *Deinococcus-Thermus* group and the green non-sulphur bacteria, which are two of the earliest branching divisions among Gram-negative bacteria (Gupta, 1998a). A key signature for the phylogenetic placement of *F. succinogenes* is found in the protein Ala-tRNA synthetase. A 4 aa insert present in this protein is found only in *Proteobacteria* and in the *Chlamydia* and CFB groups of species (Gupta, 2000a). Since this insert is also present in the *F. succinogenes* homologue, it provides strong evidence that *F. succinogenes* is related to one of these taxa. The presence of this insert also excludes *F. succinogenes* from the earlier branching spirochaetes and the cyanobacterial divisions. The distinction whether *F. succinogenes* belongs to the proteobacterial group or to the *Chlamydia*–CFB group is made on the basis of two signature sequences in CTP synthase and Hsp70 proteins that are distinctive of the proteobacterial species. The absence of these signatures in the *F. succinogenes* homologues provides evidence that this species does not belong to the proteobacterial group and places it in a similar position as the *Chlamydia*–CFB group.

The placement of *Fibrobacter* in a similar position as the *Chlamydia*–CFB group, as carried out in this work, is based on a single type species and further confirmation of this inference with additional type species would be

Fig. 6. A summary diagram showing the deductive process used to determine the branching position of species. The arrows indicate the stages where the signature sequences in different proteins examined in the present work were introduced. All groups underneath the arrows share the indicated indels and no exceptions to these were observed. The other signatures used to deduce the branching orders of various groups have been described previously (Gupta, 1998a, 2000a) and are not shown here. Monoderms (cell types bounded by a single membrane) are shown to be ancestral to the diderms (bounded by two different membranes) (Gupta, 1998a, 2000a). Based on different signatures, *F. succinogenes* is indicated to have evolved after the branching of spirochaetes and other groups preceding it, but before the branching of different divisions of *Proteobacteria*.

very helpful. However, a specific relationship of *F. succinogenes* to the *Chlamydia*-CFB group, which includes the *Bacteroides* family, is supported by the fact that, in a similar manner to *F. succinogenes*, many other members of this group are able to digest and grow on complex substrates such as cellulose, chitin and agar (Hungate, 1950; Paster *et al.*, 1994; Reichenbach, 1992; Shah, 1992; Weisburg *et al.*, 1985; Woese, 1987). *F. succinogenes*, originally known as *Bacteroides succinogenes*, was first placed in the *Bacteroidaceae* family (Holdeman *et al.*, 1984). Subsequent phylogenetic studies based on 16S rRNA indicated that *F. succinogenes* was less closely related to other species of *Bacteroides*, including the type species *Bacteroides fragilis* as compared to species belonging to other families such as *Flavobacterium heparinum* (Amann *et al.*, 1992; Montgomery *et al.*, 1988). Although, based on these observations, *F. succinogenes* was transferred to the new genus *Fibrobacter*, these studies still indicate that in comparison to the other divisions within the *Bacteria*, members of the CFB group are the closest relatives of *Fibrobacter*. The relative branching order of different species within the *Chlamydia*-CFB-*Fibrobacter* group is presently unclear. It is expected that as additional signature sequences that enable further distinction between this group of species are identified, the relationship between members of this diverse group will become clearer.

Lastly, it should be emphasized that the use of signature sequences for the phylogenetic placement of species into different groups, or to understand the relative branching orders of these groups, is not contradictory to the phylogenetic analysis based on 16S rRNA, but it complements such analyses in important respects. In our earlier work, where placement of a large number of bacterial species into different groups was carried out using the signature sequence approach, a nearly perfect correlation was observed with the assignment based on the 16S rRNA trees (Gupta, 1998a, 2000a). However, the main advantage of the signature approach lies in the fact that it allows deduction of the branching orders of the main bacterial taxa, which thus far cannot be reliably determined based on the rRNA or other gene/protein phylogenies. The signature sequences in various proteins also permit a more precise molecular definition of different bacterial groups (or means to distinguish between them) than has hitherto been possible based on the phylogenetic trees. In these regards the signature sequence approach complements the major deficiencies of the rRNA trees in clarifying/understanding the genealogy of the *Bacteria*. It should however be mentioned that although the signature sequences are able to make clear distinction between different groups, they do not provide detailed information regarding the interrelationship of various species within a given group. Such information is contained in

the phylogenetic trees. Hence, the use of signature sequences and the analysis based on phylogenetic trees provide complementary, and not contradictory, approaches for understanding the evolutionary relationships among prokaryotes.

ACKNOWLEDGEMENTS

The work was supported by supported by a research grant from the Medical Research Council (Canadian Institute of Health Research) of Canada. We are grateful to Drs C. Forsberg and J. Yanke for providing us with *F. succinogenes* DNA.

REFERENCES

- Amann, R. L., Lin, C., Key, R., Montgomery, L. & Stahl, D. A. (1992). Diversity among *Fibrobacter* isolates: towards a phylogenetic classification. *Syst Appl Microbiol* **15**, 23–31.
- Asanuma, N., Iwamoto, M. & Hino, T. (1999). Effect of the addition of fumarate on methane production by ruminal microorganisms in vitro. *J Dairy Sci* **82**, 780–787.
- Brown, J. R. & Doolittle, W. F. (1995). Root of the universal tree of life based on ancient aminoacyl-tRNA synthetase gene duplications. *Proc Natl Acad Sci U S A* **92**, 2441–2445.
- Cheng, K. J., Forsberg, C. W., Minato, H. & Costerton, J. W. (1991). Microbial ecology and physiology of feed degradation within the rumen. In *Physiological Aspects of Digestion and Metabolism in Ruminants*, pp. 595–624. Edited by T. Tsuda, Y. Sasaki & R. Kawahima. New York: Academic Press.
- Debroas, D. & Blanchart, G. (1993). Interactions between proteolytic and cellulolytic rumen bacteria during hydrolysis of plant cell wall protein. *Reprod Nutr Dev* **33**, 283–288.
- Doolittle, W. F. (1999). Phylogenetic classification and the universal tree. *Science* **284**, 2124–2128.
- Fields, M. W., Mallik, S. & Russell, J. B. (2000). *Fibrobacter succinogenes* S85 ferments ball-milled cellulose as fast as cellobiose until cellulose surface area is limiting. *Appl Microbiol Biotechnol* **54**, 570–574.
- Fraser, C. M., Gocayne, J. D., White, O. & 7 other authors (1995). The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**, 397–403.
- Galley, K. A., Singh, B. & Gupta, R. S. (1992). Cloning of HSP70 (*dnaK*) gene from *Clostridium perfringens* using a general polymerase chain reaction based approach. *Biochim Biophys Acta* **1130**, 203–208.
- Garcia-Vallve, S., Romeu, A. & Palau, J. (2000). Horizontal gene transfer of glycosyl hydrolases of the rumen fungi. *Mol Biol Evol* **17**, 352–361.
- Gokarn, R. R., Eitnan, M. A., Martin, S. A. & Eriksson, K. E. (1997). Production of succinate from glucose, cellobiose, and various cellulosic materials by the ruminal anaerobic bacteria *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*. *Appl Biochem Biotechnol* **68**, 69–80.
- Gupta, R. S. (1995). Evolution of the chaperonin families (Hsp60, Hsp10 and Tcp-1) of proteins and the origin of eukaryotic cells. *Mol Microbiol* **15**, 1–11.
- Gupta, R. S. (1998a). Protein phylogenies and signature sequences: a reappraisal of evolutionary relationships among Archaeobacteria, Eubacteria, and Eukaryotes. *Microbiol Mol Biol Rev* **62**, 1435–1491.
- Gupta, R. S. (1998b). What are archaeobacteria: life's third domain or monoderm prokaryotes related to Gram-positive bacteria? A new proposal for the classification of prokaryotic organisms. *Mol Microbiol* **29**, 695–708.
- Gupta, R. S. (2000a). The phylogeny of Proteobacteria: relationships to other Eubacterial phyla and eukaryotes. *FEMS Microbiology Reviews* **24**, 367–402.
- Gupta, R. S. (2000b). The natural relationships among prokaryotes. *CRC Crit Rev Microbiol* **26**, 111–131.
- Gupta, R. S. (2000c). Evolutionary relationships among bacteria: does 16S rRNA provide all the answers? *ASM News* **66**, 189–190.
- Gupta, R. S. & Golding, G. B. (1993). Evolution of HSP70 gene and its implications regarding relationships between archaeobacteria, eubacteria, and eukaryotes. *J Mol Evol* **37**, 573–582.
- Gupta, R. S. & Singh, B. (1992). Cloning of the HSP70 gene from *Halobacterium marismortui*: relatedness of archaeobacterial HSP70 to its eubacterial homologs and a model for the evolution of the HSP70 gene. *J Bacteriol* **17**, 4594–4605.
- Gupta, R. S., Mukhtar, T. & Singh, B. (1999). Evolutionary relationships among photosynthetic prokaryotes (*Heliobacterium chlorum*, *Chloroflexus aurantiacus*, Cyanobacteria, *Chlorobium tepidum* and Proteobacteria): implications regarding the origin of photosynthesis. *Mol Microbiol* **32**, 893–906.
- Holdeman, L. V., Kelley, R. W. & Moore, W. E. C. (1984). Family I. *Bacteroidaceae* Pribam 1933. In *Bergey's Manual of Systematic Bacteriology*, pp. 602–662. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Huang, L. & Forsberg, C. W. (1990). Cellulose digestion and cellulase regulation and distribution in *Fibrobacter succinogenes* subsp. *succinogenes* S85. *Appl Environ Microbiol* **56**, 1221–1228.
- Hugenholtz, P., Pitulle, C., Hershberger, K. L. & Pace, N. (1998). Novel division level bacterial diversity in a Yellowstone hot spring. *J Bacteriol* **180**, 366–376.
- Hungate, R. E. (1950). The anaerobic mesophilic cellulolytic bacteria. *Bacteriol Rev* **14**, 1–49.
- Iwabe, N., Kuma, K., Hasegawa, M., Osawa, S. & Miyata, T. (1989). Evolutionary relationship of archaeobacteria, eubacteria, and eukaryotes inferred from phylogenetic trees of duplicated genes. *Proc Natl Acad Sci U S A* **86**, 9355–9359.
- Lindquist, S. & Craig, E. A. (1988). The heat-shock proteins. *Annu Rev Genet* **22**, 631–677.
- Ludwig, W. & Schleifer, K. H. (1999). Phylogeny of *Bacteria* beyond the 16S rRNA Standard. *ASM News* **65**, 752–757.
- Maidak, B. L., Cole, J. R., Parker, C. T., Jr and 11 other authors (1999). A new version of the RDP (Ribosomal Database Project). *Nucleic Acids Res* **27**, 171–173.
- Montgomery, L., Flesher, B. & Stahl, D. (1988). Transfer of *Bacteroides succinogenes* (Hungate) to *Fibrobacter* gen. nov. as *Fibrobacter succinogenes* comb. nov. and description of *Fibrobacter intestinalis* sp. nov. *Int J Syst Bacteriol* **38**, 430–435.
- Olsen, G. J. & Woese, C. R. (1993). Ribosomal RNA: a key to phylogeny. *FASEB J* **7**, 113–123.
- Paster, B. J., Dewhirst, F. E., Olsen, I. & Fraser, G. J. (1994). Phylogeny of *Bacteroides*, *Prevotella*, and *Porphyromonas* spp. and related bacteria. *J Bacteriol* **176**, 725–732.
- Reichenbach, H. (1992). The Order *Cytophagales*. In *The Prokaryotes*, pp. 3631–3675. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
- Rusanganwa, E., Singh, B. & Gupta, R. S. (1992). Cloning of HSP60 (GroEL) operon from *Clostridium perfringens* using a polymerase chain reaction based approach. *Biochim Biophys Acta* **1130**, 90–94.

- Shah, R. N. (1992).** The genus *Bacteroides* and related taxa. In *The Prokaryotes*, pp. 3593–3607. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
- Shi, Y., Odt, C. L. & Weimer, P. J. (1997).** Competition for cellulose among three predominant ruminal cellulolytic bacteria under substrate-excess and substrate-limited conditions. *Appl Environ Microbiol* **63**, 734–742.
- Wang, Y., McAllister, T. A., Yanke, L. J. & Cheeke, P. R. (2000).** Effect of steroidal saponin from *Yucca schidigera* extract on ruminal microbes. *J Appl Microbiol* **88**, 887–896.
- Weimer, P. J., Waghorn, G. C., Odt, C. L. & Mertens, D. R. (1999).** Effect of diet on populations of three species of ruminal cellulolytic bacteria in lactating dairy cows. *J Dairy Sci* **82**, 122–134.
- Weisburg, W. G., Oyaizu, Y., Oyaizu, H. & Woese, C. R. (1985).** Natural relationship between *Bacteroides* and *Flavobacteria*. *J Bacteriol* **164**, 230–236.
- Woese, C. R. (1987).** Bacterial evolution. *Microbiol Rev* **51**, 221–271.
- Woese, C. R. (1998).** The universal ancestor. *Proc Natl Acad Sci USA* **95**, 6854–6859.
- Zinder, S. H. (1998).** Bacterial diversity. In *Topley and Wilson's Microbiology and Microbial Infections*, 9th edn, vol. 2, pp. 125–147. Edited by A. Balows & B. I. Duerden. London: Arnold.
-

Received 15 February 2001; revised 17 May 2001; accepted 25 May 2001.