Evaluation of the phylogenetic position of the sulfate-reducing bacterium *Thermodesulfovibrio yellowstonii* (phylum *Nitrospirae*) by means of gene order data from completely sequenced genomes

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The phylogenetic placement of *Thermodesulfovibrio yellowstonii* was investigated on the basis of gene order data from completely sequenced bacterial genomes. *T. yellowstonii* was found to share four gene arrangements characteristic of the *Proteobacteria*, *Aquificae*, *Planctomycetes*, *Spirochaetes*, *Bacteroidetes*, *Chlorobi*, *Acidobacteria*, *Verrucomicrobia* and termite group 1, the presence of which defines superphylum 2. The remaining phyla show sets of alternative gene arrangements and form superphylum 1. An analysis of conserved gene pairs showed that the overall genome organization of *T. yellowstonii* is most similar to that of *deltaproteobacteria*. Three arrangements that suggest gene translocations were identified that are likely to have occurred in a common ancestor of *T. yellowstonii* and the *Proteobacteria* exclusive of virtually all other major bacterial phyla. The translocation events suggest the closest evolutionary relationship between *T. yellowstonii* and the *Proteobacteria*.

INTRODUCTION

Thermodesulfovibrio yellowstonii is a thermophilic, sulfate-reducing bacterium that was originally isolated from thermal vent water in Yellowstone Lake (Henry *et al.*, 1994). An early phylogenetic study based on 16S rRNA gene sequence comparisons indicated that the Gram-negative *T. yellowstonii* branched deeply in the domain *Bacteria* and is classified in a distinct phylum, *Nitrospirae* (Henry *et al.*, 1994). The placement as the third deepest branch, following two other thermophiles, *Aquifex* and *Thermotoga*, supports the theory of thermophilic origins of the *Bacteria* (Achenbach-Richter *et al.*, 1987). A more recent analysis of 16S rRNA gene sequences indicated that the phylum *Nitrospirae*, including *T. yellowstonii*, branched off after the divergence of *Aquifex*, *Thermotoga*, *Chloroflexus* and the *Thermus–Deinococcus* group and then formed a cluster with *proteobacteria* (*Rhodospirillum rubrum* and *Chromatium vinosum*) and a Gram-positive bacterium, *Bacillus subtilis* (Castro *et al.*, 2000). On the other hand, another member of the phylum *Nitrospirae*, *Nitrospira marina*, was reported to be affiliated with the *Deltaproteobacteria* (Teske *et al.*, 1994) on the basis of 16S rRNA gene sequence comparison.

Although the phylogenetic position of *T. yellowstonii* has so far been inferred based on the 16S rRNA gene sequence, the

availability of genome sequence data (R. J. Dodson, A. S. Durkin, M. Wu, J. Eisen and G. Sutton, unpublished NCBI accession no. NC_011296; submitted 29 August 2008) presents new phylogenetic challenges. It is widely believed that genome trees based on a concatenation of multiple protein sequences are less susceptible to stochastic errors than those based on a single protein or gene such as the 16S rRNA gene. Genome trees reported recently showed the power of this approach, confirming accepted phylogenies and/or presenting unexpected novel phylogenetic relationships at the level of phyla as well as individual species (Brown *et al.*, 2001; Wolf *et al.*, 2001; Teeling *et al.*, 2004; Ciccarelli *et al.*, 2006; Wu & Eisen, 2008; Ward *et al.*, 2009). Concatenation of trees for individual proteins (supertree method) has also proved useful for reconstructing bacterial phylogeny in the presence of lateral gene transfer (Daubin *et al.*, 2002). While separation of the major bacterial phyla was largely improved in genome trees or supertrees, branching order at the phylum level remained poorly resolved. Another useful approach is to find conserved insertions/deletions (indels) in protein sequences. Based on a series of conserved indels found in various proteins, a linear branching order of the major bacterial phyla, *Firmicutes*→*Actinobacteria*→*Clostridium*→*Fusobacterium*→*Thermotoga*→*Deinococcus*→*Thermus*→*Chloroflexus*→*Cyanobacteria*→*Spirochaetes*→*Chlamydia*→*Cytophaga*→*Flavobacteria*→*Chlorobi*→*Aquifex*→*Proteobacteria*, has been proposed (Gupta, 1998; Gupta & Griffiths, 2002). A third approach is to compare gene orders

A list of completely sequenced bacterial genomes used in this study and results of BLAST best-hit analyses of ribosomal proteins are available as supplementary material with the online version of this paper.

or gene contents of completely sequenced genomes (Sankoff *et al.*, 1992; Wolf *et al.*, 2001; Korbel *et al.*, 2002). Although several difficulties are known in methods based on gene order or gene content (e.g. Delsuc *et al.*, 2005), they do not rely on multiple sequence alignments and, hence, can provide independent support for phylogenies inferred from aligned sequences.

In the present paper, phylogenetic relationships of *T. yellowstonii* to other bacteria were investigated on the basis of gene arrangements that are uniquely found in a group of phyla. If a gene *x* is located between genes *a* and *b*, i.e. in the order *a–x–b*, in one group, while the same gene *x* is found between genes *c* and *d*, in the arrangement *c–x–d*, in another group, then a translocation of gene *x* is thought to have occurred in a common ancestor of either group (Kunisawa, 2001, 2003). Three arrangements that suggest gene translocations were identified, and they provide empirical evidence that the thermophilic, sulfate-reducing bacterium *T. yellowstonii* and the *Proteobacteria* are in a close, probably closest, evolutionary relationship; they share common ancestry exclusive of *Acidobacteria*, *Chlamydiae*, termite group 1, *Planctomycetes*, *Spirochaetes* and *Verrucomicrobia*, after the divergence from another group of phyla that comprises *Firmicutes*, *Actinobacteria*, *Fusobacteria*, *Thermotogae*, *Chloroflexus*, *Thermus–Deinococcus* and *Dictyoglomi*.

METHODS

Gene order data. Complete genomic sequences of bacteria were downloaded from the ftp server at NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/). In order to identify orthologous relationships of genes readily among different genomes, all the protein-encoding genes were labelled systematically, using the Cluster of Orthologous Groups of proteins (COG) database, in which each homologous gene family is labelled by a unique four-digit number (Tatusov *et al.*, 1997, 2001). Protein-encoding genes present on a genome were represented as a four-digit COG number by performing the FASTA similarity search (Pearson & Lipman, 1988). In this assignment, a FASTA E-value of 10^{-4} was used as a threshold. For those genes that showed a less significant E-value, between 10^{-2} and 10^{-4} , COG numbers were assigned manually by taking account of protein length, percentage identity and gene order (Kunisawa, 2006). Thus, gene order along each genome was represented as COG order. tRNA-encoding genes were searched for by the tRNAscan-SE program (Lowe & Eddy, 1997). tRNA genes thus identified were inserted into the COG order of protein-encoding genes according to their base coordinates. rRNA genes were also put into the COG order if they were annotated in the original genomic sequence. Thus, gene order comparisons are readily carried out using the COG/tRNA/rRNA orders thus generated. There are many completely sequenced genomes with the same species name but different strains, which reveal almost identical gene orders. Therefore, in the present analysis, one arbitrarily chosen strain was taken into account, and others were neglected. A total of 425 bacterial genomes were compared (see Supplementary Table S1, available in IJSEM Online). Although *Coprothermobacter proteolyticus* is annotated as a member of the *Firmicutes* in the NCBI taxonomy, 16S rRNA gene phylogenies indicate that this bacterium forms a distinct phylum well-separated from the *Firmicutes* (Etchebehere *et al.*, 1998; Hugenholtz *et al.*, 1998). Therefore, *Coprothermobacter proteolyticus* is treated as representing a separate phylum '*Coprothermobacter*' in the present study.

Computer search. A computer search was carried out with an asymmetrical constraint on gene order so that *a–x–b* could be detected in genomes of the Gram-positive actinobacteria and firmicutes and *c–x* (or *x–d*) in Gram-negative proteobacterial genomes and vice versa. The search procedure is simple and straightforward, as described in previous papers (Kunisawa, 2003, 2006). First, assume that one gene among the complete gene set is a transposed gene, *x*, and define its neighbouring genes, *a* and *b*, on each genome. When an identical arrangement *a–x–b* is found among the *Actinobacteria/Firmicutes* and another phylum, the other arrangement, *c–x* (or *x–d*), is searched for that is conserved among the *Proteobacteria* and the other phyla, and vice versa. This procedure was repeated for all the genes that are present on the completely sequenced genomes. In order to avoid complicated assignments of gene orthology, the search was not carried out for cases where gene *x* is present in multiple copies on more than half of the genomes under comparison.

RESULTS

Dichotomy of bacterial phyla

Previous analyses of gene arrangements on bacterial genomes have suggested that the major bacterial phyla are divided into two groups (superphyla 1 and 2); one group comprises the Gram-positive bacteria (*Firmicutes* and *Actinobacteria*), *Thermotogae*, *Chloroflexi* and *Deinococcus–Thermus*, while the other includes the Gram-negative *Proteobacteria*, *Aquificae*, *Planctomycetes*, *Spirochaetes*, *Bacteroidetes* and *Chlorobi* (Kunisawa, 2006). Since then, many more bacterial genomes have been completely sequenced, which enables phylogenomic studies of new phyla, i.e. *Dictyoglomi*, *Acidobacteria*, termite group I, *Verrucomicrobia* and *Nitrospirae*. I first verified the dichotomy of bacterial phyla in the light of the 425 currently available bacterial genomes, and then examined to which superphylum *T. yellowstonii* belongs. I carried out a computer search for sets of alternative gene arrangements, *a–x–b* in one group of phyla and *c–x* (or *x–d*) in the other group for the same gene *x*. Four such arrangements were identified by the present computer search.

The first example is found around a glycine-tRNA gene with the anticodon sequence TCC (Gtcc). This Gly-tRNA gene is present in a single copy on most genomes; if present in multiple copies, its copy number per genome is shown in brackets following the species name in Fig. 1(a). Contiguous genes are connected by dashes, while split segments are interrupted by forward slashes. Homologous genes are vertically aligned. As seen in Fig. 1(a), Gtcc is located between a proline-tRNA gene with the anticodon TGG (Ptgg) and an arginine-tRNA gene with the anticodon TCT (Rtct) in 36 of 40 completely sequenced actinobacterial genomes. The numerator in parentheses following the phylum name represents the number of genomes showing conserved gene arrangements, and the denominator is the total number of compared genomes belonging to that phylum. The adjacency of Gtcc to Ptgg and/or Rtct can also be found in the *Firmicutes*,

Synergistetes, *Fusobacteria*, *Deinococcus-Thermus*, *Dictyoglomi* and *Thermotogae*. Some typical gene arrangements are shown for each phylum. For example, the genome of *Bacillus halodurans* harbours three copies of *Gtcc*, two of which are adjacent to *Ptgg* or *Rtct*. Nine out of 23 genomes from the *Bacillales* show similar gene arrangements. By contrast, *Gtcc* is not found between *Ptgg* and *Rtct* in the *Proteobacteria* but is located between a tyrosine-tRNA gene with the anticodon sequence GTA (Ygta) and a threonine-tRNA gene with the anticodon GGT (Tggt). This latter gene arrangement can be found in the *Aquificae*, *Acidobacteria*, *Planctomycetes*, *Bacteroidetes*, termite group 1 and '*Coprothermobacter*' as well as *T. yellowstonii* (*Nitrospirae*). Members of the *Spirochaetes*, *Chlorobi*, *Verrucomicrobia*, *Chlamydiae* and *Cyanobacteria* do not reveal either of these gene arrangements. A few members of the *Clostridia* (*Firmicutes*) show a mixed arrangement; in *Finegoldia magna*, the *Gtcc* gene is located adjacent to both *Rtcc* and *Ygta*, and similar arrangements are found in *Alkaliphilus metalliredigens* and *Alkaliphilus oremlandii*, around one of the *Gtcc* genes present in four and five copies per genome, respectively, as shown within brackets in Fig. 1(a). Thus, except for a few clostridia, the major bacterial phyla are likely to be divided into two groups on the basis of gene arrangement around the *Gtcc* gene.

Similar alternative gene arrangements were identified around the *nusB* gene (COG0781), which encodes a transcription termination factor. In the *Firmicutes*, *Synergistetes*, *Actinobacteria*, *Fusobacteria*, *Deinococcus-Thermus*, *Chloroflexi*, *Dictyoglomi* and *Thermotogae*, *nusB* is located in the neighbourhood of *efp* (COG0231), *yqhY* (COG1302), *fold* (COG0190) and *xseA* (COG1722). In contrast, *nusB* is adjacent to *ribH* (COG0054) in *T. yellowstonii* and members of the *Proteobacteria*, *Verrucomicrobia*, *Spirochaetes*, *Acidobacteria* and *Aquificae* (Fig. 1b). In Fig. 1, for maximum alignment of gene order, some contiguous ORFs are represented by dots.

A conserved hypothetical protein-encoding gene designated *yvyD* (COG1544) is located adjacent to *comFC* (COG1040) and/or to *secA* (COG0653) in the *Firmicutes*, '*Coprothermobacter*', *Actinobacteria*, *Dictyoglomi* and *Chloroflexi* (Fig. 1c). In *T. yellowstonii*, *yvyD* is located between *rpoN* (COG1508) and *yhbJ* (COG1660), and similar arrangements are found in members of the *Proteobacteria*, termite group 1, *Spirochaetes*, *Acidobacteria*, *Planctomycetes* and *Aquificae*.

The computer search identified a fourth set of alternative gene arrangements around *lepA*, encoding a membrane GTPase. As shown in Fig. 1(d), the *lepA* gene (COG0481) is present between *rpsT* (COG0268) and *hemN* (COG0635) in the *Firmicutes*, *Synergistetes*, *Actinobacteria* and *Dictyoglomi*. In Fig. 1, genes designated with an asterisk, i.e. *rpsT* (COG0268) and *degQ* (COG0265), are located on the strand complementary to that of *lepA*, *hemN* and *lepB*, and are transcribed from right to left. In *T. yellowstonii*

and members of the *Proteobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chlorobi* and *Cyanobacteria*, the arrangement *rpsT*–*lepA*–*hemN* is not found, but another arrangement, *degQ* (COG0265)–*lepA* (COG0481)–*lepB* (COG0681), is observed. Two members of the *Clostridia* exceptionally show a mixed arrangement, *lepB*–(ORF)–*lepA*–*hemN*.

The phylum–gene arrangement relationships thus obtained are summarized in Table 1, in which gene arrangements of Gram-positive firmicutes/actinobacteria are designated '+', while proteobacterial arrangements are denoted '−'. Here, note that all the bacteria grouped in '+' (−) are not necessarily Gram-positive (−negative); for instance, *Dictyoglomus thermophilum* (phylum *Dictyoglomi*, grouped into '+') has been reported to stain Gram-negative (Saiki *et al.*, 1985). Since the exceptional arrangements around *Gtcc* and *lepA* are only found in a few members of the *Clostridia*, they are not taken into account in the table. Although neighbouring gene pairs that are conserved among different genomes are known to interact physically at the protein level (Dandekar *et al.*, 1998), such physical interactions seem unlikely among the gene products listed in Fig. 1. It is interesting to note that the patterns of division into '+' or '−' in Table 1 are almost consistent, suggesting an evolutionary event. Thus, bacterial phyla appear to reveal a fundamental split into two groups (superphyla): one comprises the *Firmicutes*, *Synergistetes*, *Actinobacteria*, *Thermotogae*, *Chloroflexi*, *Deinococcus-Thermus*, *Fusobacteria* and *Dictyoglomi* and the other consists of the *Proteobacteria*, *Aquificae*, *Planctomycetes*, *Spirochaetes*, *Bacteroidetes*, *Chlorobi*, *Acidobacteria*, *Verrucomicrobia*, termite group 1 and *Nitrospirae* (*T. yellowstonii*). It is to be noted that an identical distribution of '−' is observed between *T. yellowstonii* and the *Proteobacteria*, suggesting their close evolutionary relationship.

Conserved gene-pair analysis

In order to assess evolutionary relationships of *T. yellowstonii* to other phyla at the level of overall genome organization, a conserved gene-pair analysis (Wolf *et al.*, 2001; Korbel *et al.*, 2002) was performed. Here, a conserved gene pair is defined as a pair of genes that are adjacent to each other in the same order on both genomes under comparison. For the sake of ready comparison of genome organization, we have generated reduced gene orders in which genes shared by the two genomes under pairwise comparison were retained, while genes present in only one genome were neglected. Using gene orders generated in this way, the fraction f_c of conserved gene pairs was evaluated in comparisons of *T. yellowstonii* to other bacteria. Values of f_c averaged over each phylum are listed in Table 2. The *Mollicutes* (*Firmicutes*) were neglected in this evaluation because of the extreme smallness of their genomes. As can be seen in Table 2, the genome organization of *T. yellowstonii* is most similar to those of the *Delta-proteobacteria* (0.238), followed by the *Acidobacteria*

(a)

<i>Thermotogae</i> (7/7)			
<i>Thermotoga maritima</i>	/Gtcc--Rtct--Hgtg		
<i>Thermosipho melanesiensis</i>	Ptgg--Gtcc--Rtct--Hgtg		
<i>Dictyoglomi</i> (1/1)			
<i>Dictyoglomus thermophilum</i>	Ptgg--Gtcc--Rtct--Hgtg		
<i>Deinococcus-Thermus</i> (3/3)			
<i>Deinococcus geothermalis</i>	Ptgg--Gtcc--Rtct/		
<i>Thermus thermophilus</i>	Ptgg--Gtcc--Rtct/		
<i>Fusobacteria</i> (1/1)			
<i>Fusobacterium nucleatum</i>	Ptgg--Gtcc--Rtct--Hgtg		
<i>Actinobacteria</i> (36/40)			
<i>Rubrobacter xylanophilus</i>	Ptgg--Gtcc/		
<i>Corynebacterium efficiens</i>	/Gtcc--Ptgg		
<i>Synergistetes</i> (1/2)			
<i>Syntrophomonas wolfei</i>	/Gtcc--Rtct--Hgtg		
<i>Firmicutes – Clostridia</i> (19/24)			
<i>Symbiobacterium thermophilum</i>	Ptgg--Gtcc--Rtct--Hgtg		
<i>Pelotomaculum thermopropionicum</i>	/Gtcc--Rtct/		
<i>Clostridium acetobutylicum</i> [4]	Ptgg--Gtcc--Rtct--Hgtg		
<i>Heliobacterium modesticaldum</i>	/Gtcc-----Hgtg		
<i>Firmicutes – Bacillales</i> (9/23)			
<i>Bacillus halodurans</i> [3]	Ptgg--Gtcc		
<i>Bacillus halodurans</i> [3]	Gtcc--Rtcc		
<i>Firmicutes – Mollicutes</i> (4/18)			
<i>Acholeplasma laidlawii</i>	Ptgg--Gtcc		
<i>Aquificae</i> (3/3)			
' <i>Aquifex aeolicus</i> '		Ttgt--Ygta--Gtcc--Tggc--tufA	
<i>Sulfurihydrogenibium</i> sp.	Ptgg-----Rtct/	Ttgt--Ygta--Gtcc--Tggc--tufA	
<i>Nitrospirae</i> (1/1)			
<i>Thermodesulfobivrio yellowstonii</i>		Ttgt--Ygta--Gtcc--Tggc--tufA	
<i>Acidobacteria</i> (1/2)			
<i>Acidobacteria bacterium</i>		Ttgt--Ygta--Gtcc--Tggc--tufA	
<i>Planctomycetes</i> (1/1)			
<i>Rhodopirellula baltica</i>		Ttgt--Ygta--Gtcc--Tggc--tufA	
<i>Bacteroidetes</i> (10/12)			
<i>Bacteroides thetaiotaomicron</i> [2]		Ttgt--Ygta--Gtcc--Tggc--tufA	
<i>Cytophaga hutchinsonii</i>	Ptgg-----Rtct/	Ygta--Gtcc--Tggc--tufA	
Termite group 1 (1/1)			
' <i>Elusimicrobium minutum</i> '		Gtcc--Tggc--tufA	
' <i>Coprothermobacter</i> ' (1/1)			
<i>Coprothermobacter proteolyticus</i>		Ygta--Gtcc--Tggc--tufA	
<i>Proteobacteria – Deltaproteobacteria</i> (18/19)			
<i>Geobacter sulfurreducens</i>	Ptgg-----Rtct--Hgtg/	Ttgt--Ygta--Gtcc--Tggc--tufA	
<i>Proteobacteria – Epsilonproteobacteria</i> (13/13)			
<i>Campylobacter jejuni</i>	Ptgg-----Rtct--Hgtg/	Ttgt--Ygta--Gtcc--Tggc--tufA	
<i>Proteobacteria – Alphaproteobacteria</i> (48/66)			
<i>Parvibaculum lavamentivorans</i>	Ptgg-----Rtct/	Ygta--Gtcc	
<i>Proteobacteria – Betaproteobacteria</i> (33/33)			
<i>Neisseria meningitidis</i>		Ygta--Gtcc--Tggc--tufA	
<i>Proteobacteria – Gammaproteobacteria</i> (79/80)			
<i>Haemophilus influenzae</i>		Ttgt--Ygta--Gtcc--Tggc--tufA	
Exceptions in <i>Firmicutes – Clostridia</i>			
<i>Fingoldia magna</i>	Ttgt--Ygta--Gtcc--Rtct		
<i>Alkaliphilus metalliredigens</i> [5]	Ttgt-----Gtcc--Rtct		
<i>Alkaliphilus oremlandii</i> [4]	Ttgt-----Gtcc--Rtct		
<i>Alkaliphilus oremlandii</i> [4]	Ttgt--Ygta--Gtcc/		

(b)

<i>Thermotogae</i> (7/7)	
<i>Thermotoga maritima</i>	0231-----1302--0781-0190--1570--1722--1154
<i>Dictyoglomi</i> (1/1)	
<i>Dictyoglomus thermophilum</i>	/1302-0781-----1154
<i>Chloroflexi</i> (1/6)	
<i>Herpetosiphon aurantiacus</i>	/1302-0781/
<i>Deinococcus-Thermus</i> (3/3)	
<i>Thermus thermophilus</i>	0231--0511--0439--1302--0781-0190/
<i>Fusobacteria</i> (1/1)	
<i>Fusobacterium nucleatum</i>	/1302-0781/
<i>Actinobacteria</i> (39/40)	
<i>Rubrobacter xylanophilus</i>	0231-----0781/
<i>Synergistetes</i> (2)	
<i>Syntrophomonas wolfei</i>	/1302-0781-0190--1570--1722
<i>Firmicutes – Clostridia</i> (24/24)	
<i>Symbiobacterium thermophilum</i>	/1302-0781-0190--1570--1722
<i>Firmicutes – Bacillales</i> (23/23)	
<i>Listeria monocytogenes</i>	0231--0511--0439--1302--0781-0190--1570--1722
<i>Firmicutes – Lactobacillales</i> (27/27)	
<i>Lactobacillus plantarum</i>	0231-----1302--0781-0190--1570--1722
<i>Firmicutes – Mollicutes</i> (8/18)	
<i>Mycoplasma genitalium</i>	0231-----0781/
<i>Acholeplasma laidlawii</i>	/0781-----1570--1722
	<i>efp accB accC yqhY nusB folD xseA xseB dxs</i>
<i>Aquificae</i> (2/3)	
<i>'Aquifex aeolicus'</i>	/0054-0781/
<i>Nitrospirae</i> (1/1)	
<i>Thermodesulfobacterium yellowstonii</i>	/0054-0781/
<i>Acidobacteria</i> (2/2)	
<i>Acidobacterium bacterium</i>	/0054-0781/
<i>Spirochaetes</i> (2/10)	
<i>Leptospira interrogans</i>	/0054-0781/
<i>Verrucomicrobia</i> (3/3)	
<i>Opitutus terrae</i>	/0054-0781/
<i>Proteobacteria – Deltaproteobacteria</i> (18/19)	
<i>Geobacter sulfurreducens</i>	/0054-0781/
<i>Proteobacteria – Epsilonproteobacteria</i> (13/13)	
<i>Campylobacter jejuni</i>	/0054-0781/
<i>Proteobacteria – Alphaproteobacteria</i> (42/66)	
<i>Mesorhizobium loti</i>	/0054-0781/
<i>Proteobacteria – Betaproteobacteria</i> (33)	
<i>Neisseria meningitidis</i>	/0054-0781/
<i>Proteobacteria – Gammaproteobacteria</i> (72/80)	
<i>Haemophilus influenzae</i>	/0054-0781/
	<i>ribH nusB</i>

(0.217). Table 2 also lists the mean fraction of shared genes, f_s . The gene content of *T. yellowstonii* is most similar to those of the *Aquificae* ($f_s=0.835$), whereas no close similarity at the sequence level was found between them in BLAST analyses, as discussed below. Therefore, the similarity in gene content might represent thermophilic properties of those bacteria rather than their phylogenetic relationship.

Identification of gene arrangements that suggest a close phylogenetic relationship of *T. yellowstonii* to the *Proteobacteria*

My computer search has identified the following three gene arrangements, each of which suggests a gene translocation that has occurred in a common ancestor of *T. yellowstonii* and the *Proteobacteria* exclusive of other phyla.

(c)

The *asd* gene (COG0136) coding for aspartate-semialdehyde dehydrogenase is located in the neighbourhood of *dapB* (COG0289), *lysC* (COG0527) and *dapA* (COG0329) in most members of superphylum 1, i.e. the *Thermotogae*, *Dictyoglomi*, *Chloroflexi*, *Actinobacteria*, *Synergistetes* and *Firmicutes*, and three members of superphylum 2, *Acidobacteria*, *Chlamydiae* and termite group 1. In contrast, in *T. yellowstonii* and the *Proteobacteria*, *asd* is adjacent to *leuD* (COG0066) and *leuB* (0473), as shown in Fig. 2(a). On the basis of these arrangements, it is likely that the arrangement *dapB*–*asd*–*lysC* (or other similar arrangements) was ancestral, and that the other arrangement *leuD*–*leuB*–*asd* was derived during evolution by a gene translocation of *asd* in a common ancestor of *T. yellow-*

stonii and the *Proteobacteria* exclusive of the *Thermotogae*, *Dictyoglomi*, *Chloroflexi*, *Actinobacteria*, *Synergistetes*, *Firmicutes*, *Acidobacteria*, *Chlamydiae* and termite group 1. The common ancestry of *T. yellowstonii* and the *Proteobacteria* thus suggested implies their close evolutionary relationship.

Similarly, another translocation of the *glmU* gene (COG1207) encoding *N*-acetylglucosamine-1-phosphate uridylyltransferase is likely to have occurred in a common ancestor of *T. yellowstonii* and the *Proteobacteria*; *glmU* is located between *atpD* (COG0055)–*atpC* (COG0355) and *glmS* (COG0449)–COG2928 in *T. yellowstonii* and the *Proteobacteria* while, in most members of superphylum 1

Fig. 1. Alternative gene arrangements around a glycine-tRNA gene with the anticodon sequence TCC (Gtcc) (a), *nusB* (COG0781) (b), *yvyD* (COG1544) (c) and *lepA* (COG0481) (d). Protein-encoding genes are represented by four-digit COG numbers and genes encoding tRNAs are designated using the one-letter symbol for the cognate amino acid and the anticodon sequence in lower-case letters. Homologous genes are aligned vertically. Contiguous genes are connected by dashes, while split segments are interrupted by slashes. Genes with an asterisk are encoded on the complementary strand, and are transcribed from right to left; other genes are transcribed from left to right. For maximum alignment, some ORFs are represented by dots. The numerator in parentheses following the phylum name represents the number of genomes showing conserved gene arrangements and the denominator is the total number of compared genomes belonging to that phylum. Gene copy number per genome is shown in square brackets when greater than one.

(i.e. *Thermotogae*, *Dictyoglomi*, *Chloroflexi*, *Actinobacteria*, *Synergistetes*, *Firmicutes* and '*Coprothermobacter*') and three phyla belonging to superphylum 2 (*Planctomycetes*, *Spirochaetes* and termite group 1), *glmU* is located between *ispA* (COG1947)–(Qttg) and *prsA* (COG0462) (Fig. 2b).

The third example can be observed in gene arrangements around *psd* (COG0688) and *pssA* (COG1183), respectively encoding phosphatidylserine decarboxylase and phosphatidylserine synthase. The two-gene cluster is located adjacent to *moeA* (COG0303) or *dedA* (COG0568) in the

Table 1. Dichotomy of major bacterial phyla based on gene arrangements

+, Arrangement similar to the *Firmicutes/Actinobacteria*; –, proteobacterial arrangement. NA, No data available.

Phylum	Gene arrangement			
	Gtcc	nusB	yvyD	lepA
Superphylum 1				
<i>Firmicutes</i>	+	+	+	+
<i>Synergistetes</i>	+	+	+	+
<i>Actinobacteria</i>	+	+	+	+
<i>Thermotogae</i>	+	+		
<i>Chloroflexi</i>	+	+	+	
<i>Deinococcus–Thermus</i>	+	+		
<i>Fusobacteria</i>	+	+		
<i>Dictyoglomi</i>	+	+	+	+
' <i>Coprothermobacter</i> '	–	NA	+	+
Superphylum 2				
<i>Proteobacteria</i>	–	–	–	–
<i>Aquificales</i>	–	–	–	
<i>Planctomycetes</i>	–		–	
<i>Spirochaetes</i>		–	–	
<i>Bacteroidetes</i>	–			–
<i>Chlorobi</i>				–
<i>Acidobacteria</i>	–	–	–	
<i>Verrucomicrobia</i>		–		–
Termite group 1	–		–	
<i>Nitrospirae</i>	–	–	–	–
<i>Cyanobacteria</i>				–

Actinobacteria, *Synergistetes*, *Firmicutes*, *Spirochaetes* and *Verrucomicrobia*, as shown in Fig. 2(c). In *T. yellowstonii* and *Proteobacteria*, the cluster is instead found between *ilvC* (COG0059) and *leuA* (COG0119), suggesting a translocation of the *psd–pssA* cluster in a common ancestor of *T. yellowstonii* and the *Proteobacteria* after the divergence of the *Actinobacteria*, *Synergistetes*, *Firmicutes*, *Spirochaetes* and *Verrucomicrobia*.

Combining these results, a common ancestor of *T. yellowstonii* and the *Proteobacteria* is inferred to have branched off from the trunk of superphylum 2 comprising the *Acidobacteria*, *Chlamydiae*, termite group 1, *Planctomycetes*, *Spirochaetes* and *Verrucomicrobia*, after the divergence of superphylum 1. In this way, the three sets of gene arrangements identified here provide empirical evidence of a close (probably closest) evolutionary relationship between *T. yellowstonii* and the *Proteobacteria*.

DISCUSSION

This study was undertaken to clarify the phylogenetic placement of *T. yellowstonii* at the phylum level on the basis of a new phylogenetic method using gene orders of completely sequenced genomes. I first showed that *T. yellowstonii* shares four gene arrangements characteristic of

Table 2. Fractions of conserved gene pairs and shared genes in comparison with *T. yellowstonii*

The number of analysed genomes belonging to each phylum/class is shown in parentheses.

Phylum	f_c	f_s
<i>Thermotogae</i> (7)	0.157	0.696
<i>Dictyoglomi</i> (1)	0.193	0.696
<i>Chloroflexi</i> (6)	0.177	0.619
<i>Deinococcus–Thermus</i> (3)	0.156	0.664
<i>Fusobacteria</i> (1)	0.153	0.570
<i>Actinobacteria</i> (40)	0.159	0.504
<i>Synergistetes</i> (2)	0.204	0.696
<i>Firmicutes</i>		
<i>Clostridia</i> (24)	0.194	0.611
<i>Bacillales</i> (22)	0.167	0.531
<i>Lactobacillales</i> (27)	0.139	0.526
<i>Coprothermobacter</i> (1)	0.180	0.718
<i>Cyanobacteria</i> (15)	0.103	0.640
<i>Aquificae</i> (3)	0.143	0.835
<i>Acidobacteria</i> (2)	0.217	0.533
<i>Planctomycetes</i> (1)	0.150	0.523
<i>Chlorobi</i> (9)	0.164	0.764
<i>Bacteroidetes</i> (12)	0.160	0.573
<i>Spirochaetes</i> (10)	0.187	0.584
<i>Chlamydiae</i> (6)	0.161	0.574
<i>Verrucomicrobia</i> (3)	0.179	0.678
Termite group 1 (1)	0.174	0.703
<i>Proteobacteria</i>		
<i>Deltaproteobacteria</i> (19)	0.238	0.656
<i>Epsilonproteobacteria</i> (13)	0.162	0.757
<i>Alphaproteobacteria</i> (66)	0.175	0.594
<i>Betaproteobacteria</i> (32)	0.211	0.590
<i>Gammaproteobacteria</i> (80)	0.196	0.568

the *Proteobacteria*, *Aquificae*, *Planctomycetes*, *Spirochaetes*, *Bacteroidetes*, *Chlorobi*, *Acidobacteria*, *Verrucomicrobia* and termite group 1, which form superphylum 2. The remaining phyla show a set of alternative gene arrangements and form superphylum 1 (Table 1). Second, the fraction of conserved gene pairs showed that the overall genome organization of *T. yellowstonii* is most similar to those of the *Deltaproteobacteria* (Table 2). Third, I have identified three arrangements that suggest gene translocations that are likely to have occurred in a common ancestor of *T. yellowstonii* and the *Proteobacteria* exclusive of virtually all other major bacterial phyla (Fig. 2), implying the closest evolutionary relationship between *T. yellowstonii* and the *Proteobacteria*. This conclusion is not surprising, since a close affinity of another genus, *Nitrospira*, belonging to the phylum *Nitrospirae* to the *Deltaproteobacteria* was suggested previously on the basis of 16S rRNA gene sequence comparison (Teske *et al.*, 1994).

In order to assess evolutionary affinities of *T. yellowstonii* to other bacterial phyla at the protein sequence level, a BLAST best-hit analysis (Altschul *et al.*, 1997) was performed (the results are provided in Supplementary Table S2).

(a)

<i>Thermotogae</i>		
<i>Thermotoga maritima</i>	0136	-----0329--0289.-0527
<i>Fervidobacterium nodosum</i>	0136	-----0329--0289.-0527
<i>Dictyoglomi</i>		
<i>Dictyoglomus thermophilum</i>	0136	-0527
<i>Chloroflexi</i>		
' <i>Dehalococcoides ethenogenes</i> '	0289	--0136
<i>Roseiflexus castenholzii</i>	0527	-----0136
<i>Actinobacteria</i>		
<i>Rubrobacter xylanophilus</i>	0527	-----0136
<i>Mycobacterium tuberculosis</i>	0527	-----0136
<i>Synergistetes</i>		
<i>Syntrophomonas wolfei</i>	0289	.-0136-----0329
<i>Caldicellulosiruptor saccharolyticus</i>		0136-----0329--0289--0527
<i>Firmicutes – Clostridia</i>		
<i>Symbiobacterium thermophilum</i>	0289	.-0136--0527--0329
<i>Clostridium perfringens</i>		0136-----0329--0289
<i>Firmicutes – Bacillales</i>		
<i>Bacillus subtilis</i>		0136--0527--0329
<i>Listeria monocytogenes</i>		0136--0527--0329
<i>Staphylococcus aureus</i>	0527	-----0136-----0329--0289
<i>Exiguobacterium sibiricum</i>	0527	-----0136-----0329--0289
<i>Firmicutes – Lactobacillales</i>		
<i>Lactobacillus fermentum</i>	0329	-----0289.-0136
<i>Oenococcus oeni</i>	0329	-----0289.-0136
<i>Firmicutes – Mollicutes</i>		
<i>Acholeplasma laidlawii</i>	0527	-----0136-----0329--0289
<i>Acidobacteria</i>		
<i>Acidobacteria bacterium</i>		0136--0527-----0289--0329
' <i>Solibacter usitatus</i> '		0136--0527-----0289--0329
<i>Chlamydiae</i>		
<i>Chlamydophila felis</i>	0289	--0136--0527--0329
<i>Chlamydia pneumoniae</i>	0289	--0136--0527--0329
<i>Termite group 1</i>		
' <i>Elusimicrobium minutum</i> '	0329--0527	-----0136-----0289
	dapA lysC dapB asd	lysC dapA dapB dapA
<i>Nitrospirae</i>		
<i>Thermodesulfovibrio yellowstonii</i>	0066--0473	--0136--0192
<i>Proteobacteria – Deltaproteobacteria</i>		
<i>Geobacter sulfurreducens</i>	0066--0473	--0136
<i>Geobacter lovleyi</i>	0192	-----0473--0136
<i>Pelobacter propionicus</i>	0192	-----0473--0136
<i>Syntrophobacter fumaroxidans</i>	0066--0473	--0136
<i>Proteobacteria – Alphaproteobacteria</i>		
<i>Parvibaculum lavamentivorans</i>	0066--0473	--0136
<i>Brucella melitensis</i>	0473	.-0136
<i>Ehrlichia ruminantium</i>		0136--0192*
<i>Proteobacteria – Betaproteobacteria</i>		
<i>Ralstonia solanacearum</i>	0066--0473	--0136
<i>Methylobacillus flagellatus</i>	0066--0473	--0136
<i>Proteobacteria – Gammaproteobacteria</i>		
<i>Pseudomonas syringae</i>	0066--0473	--0136
<i>Marinomonas sp.</i>	0066--0473	--0136
	metK leuD leuB asd	metK

(b)

<i>Thermotogae</i>		
<i>Thermotoga maritima</i>	1207	-0462--1825--0193
<i>Fervidobacterium nodosum</i>	1207	-0462--1825--0193
<i>Dictyoglomi</i>		
<i>Dictyoglomus thermophilum</i> [2]	Qttg	1207--0462--1825
<i>Chloroflexi</i>		
<i>Roseiflexus castenholzii</i>	Qttg	1207--0462
<i>Actinobacteria</i>		
<i>Rubrobacter xylanophilus</i>	1947--Qttg	1207--0462--1825--0193
<i>Arthrobacter aurescens</i>	1947.-Qttg	1207--0462.-1825--0193
<i>Synergistetes</i>		
<i>Syntrophomonas wolfei</i>	1947.-----	1207--0462--1825--0193
<i>Firmicutes – Clostridia</i>		
<i>Symbiobacterium thermophilum</i>		1207--0462-----0193
<i>Moorella thermoacetica</i>	1947.-----	1207--0462.------0193
<i>Firmicutes – Bacillales</i>		
<i>Bacillus subtilis</i>	1947.-----	1207--0462--1825--0193
<i>Staphylococcus aureus</i>	1947.-----	1207--0462--1825--0193
<i>Firmicutes – Lactobacillales</i>		
<i>Lactobacillus sakei</i>	1947.-----	1207--0462
<i>Pediococcus pentosaceus</i>		1207.-0462
'Coprothermobacter'		
<i>Coprothermobacter proteolyticus</i>		1207--0462--1825--0193
<i>Planctomycetes</i>		
<i>Rhodopirellula baltica</i>		1207--0462
<i>Spirochaetes</i>		
<i>Leptospira interrogans</i>	1947--Qttg	1207--0462--1825--0193
<i>Leptospira biflexa</i>	1947--Qttg	1207--0462--1825--0193
Termite group 1		
' <i>Elusimicrobium minutum</i> '		1207--0462--1825--0193
	<i>ispA tRNA glmU prsA rply pth</i>	
<i>Nitrospirae</i>		
<i>Thermodesulfobacterium yellowstonii</i>	2928-----	1207--0449
<i>Proteobacteria – Deltaproteobacteria</i>		
<i>Desulfobacterium desulfuricans</i>	0055--0355.-	1207
<i>Desulfococcus oleovorans</i>	0055--0355-	1207
<i>Proteobacteria – Alphaproteobacteria</i>		
<i>Mesorhizobium loti</i>		1207.-0449--2928
<i>Sinorhizobium meliloti</i>		1207--0449.-2928
<i>Proteobacteria – Betaproteobacteria</i>		
<i>Chromobacterium violaceum</i>	0055--0355-	1207--0449
<i>Thiobacillus denitrificans</i>	0055--0355-	1207--0449
<i>Proteobacteria – Gammaproteobacteria</i>		
<i>Pseudomonas putida</i>	0055--0355-	1207--0449
<i>Xylella fastidiosa</i>	0055--0355.-	1207
	<i>atpD atpC glmU glmS orf</i>	

Sequence data of 52 ribosomal proteins from *T. yellowstonii* were used as the query sequences. Since ribosomal proteins function as components of a large macromolecular complex, their genes are unlikely to undergo lateral gene transfer with a high probability (Jain *et al.*, 1999). The best-

hit sequence was searched for against the KEGG GENES database, which collects and stores sequences translated from completely sequenced genomes and is available at GenomeNet (<http://www.genome.ad.jp/>). The BLAST analysis showed phylogenetic affinities of *T. yellowstonii* both to

(c)

Fig. 2. Gene translocations that are likely to have occurred in a common ancestor of *T. yellowstonii* and the *Proteobacteria*. Transposed genes are boxed. (a) *asd* (COG0136), (b) *glmU* (COG1207) and (c) *psd* (COG0688)–*pssA* (COG1183).

the *Clostridia* (*Firmicutes*) and to the *Proteobacteria*: 18 best-hit sequences from the *Clostridia*, 17 best hits from the *Proteobacteria* and three best hits from the *Synergistetes*, *Thermotogae*, *Cyanobacteria* and *Dictyoglomi*. Thus, the close phylogenetic affinity of *T. yellowstonii* to the *Proteobacteria* obtained from gene arrangement analyses is compatible with amino acid sequence comparisons, although the *Clostridia* are also assigned as another close relative. This assignment of the *Clostridia* seems to represent a limitation inherent in the BLAST best-hit analysis; it is known that genes that appear to be the most similar based on BLAST hits are often not phylogenetically the closest relatives (Koski & Golding, 2001).

The gene arrangements compared in Fig. 1 suggest a dichotomic division of bacterial phyla into two groups, superphyla 1 and 2. Table 1 shows a consistent grouping of the major phyla except for ‘*Coprothermobacter*’. A similar partition of bacterial phyla has been reported on the basis

of domain architectures of RNA polymerase subunits RpoB, RpoC and RpoD; three distinct large inserts of more than 100 amino acids are shared by the *Proteobacteria*, *Aquificae*, *Chlamydiae*, *Spirochaetes*, *Chlorobi* and *Planctomycetes*, while the *Firmicutes*, *Actinobacteria*, *Cyanobacteria*, *Thermus-Deinococcus*, *Thermotogae* and *Chloroflexi* all lack these inserts (Gupta, 2003; Iyer *et al.*, 2004). Thus, these two patterns are almost identical except for the *Cyanobacteria*, which are included in superphylum 2 by the sole criterion of the gene arrangements around *lepA* (see Table 1). Furthermore, these partitions of bacterial phyla into two groups are generally compatible with the topologies of genome trees of concatenated protein sequences (Iyer *et al.*, 2004; Wu & Eisen, 2008). A genome tree reconstructed quite recently using a concatenated dataset of 31 housekeeping proteins also confirms this grouping (Ward *et al.*, 2009); the *Proteobacteria*, *Acidobacteria*, *Chlorobi*, *Bacteroidetes*, *Spirochaetes* and *Chlamydiae* form a distinct clade with a

high bootstrap probability of 81 %, while its sister clade comprises the *Actinobacteria*, *Firmicutes*, *Fusobacteria*, *Chloroflexi*, *Cyanobacteria*, *Thermotogae*, *Aquificae* and *Deinococcus-Thermus*. These patterns of congruence strongly suggest that the divisions reflect a fundamental split in bacterial evolution rather than lateral gene transfers within each superphylum. It may be reasonable to consider that those phyla positioned near the boundary of the two superphyla tend to be grouped differently depending on which translocations or insertions are used as the criteria of partition. The inconsistencies found for the *Cyanobacteria* and for '*Coprothermobacter*' might be understood in this way. Although a close evolutionary relationship between the *Chlamydiae* and *Verrucomicrobia* has been proposed (Wagner & Horn, 2006; Griffiths & Gupta, 2007), the present gene arrangement analyses provided no information about the phylogenetic placement of the *Chlamydiae*. The phylogenetic placement of the *Aquificae* remains puzzling. Gene-order, gene-content and indel analyses all suggest a late emergence of this phylum (Wolf *et al.*, 2001; Korbel *et al.*, 2002; Iyer *et al.*, 2004; Griffiths & Gupta, 2004; Kunisawa, 2006), while phylogenetic trees based on the 16S rRNA gene and concatenated protein sequences reveal a basal early branching phylum (Hugenholtz *et al.*, 1998; Wolf *et al.*, 2001; Coenye & Vandamme, 2004; Ciccarelli *et al.*, 2006; Wu & Eisen, 2008; Ward *et al.*, 2009). In any case, the close evolutionary relationship between *T. yellowstonii* and the *Proteobacteria* indicates that thermophily is likely to have been acquired on some branches of bacterial evolution.

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