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Genome Diversity of Spore-Forming *Firmicutes*

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ABSTRACT Formation of heat-resistant endospores is a specific property of the members of the phylum *Firmicutes* (low-G+C Gram-positive bacteria). It is found in representatives of four different classes of *Firmicutes*, *Bacilli*, *Clostridia*, *Erysipelotrichia*, and *Negativicutes*, which all encode similar sets of core sporulation proteins. Each of these classes also includes non-spore-forming organisms that sometimes belong to the same genus or even species as their spore-forming relatives. This chapter reviews the diversity of the members of phylum *Firmicutes*, its current taxonomy, and the status of genome-sequencing projects for various subgroups within the phylum. It also discusses the evolution of the *Firmicutes* from their apparently spore-forming common ancestor and the independent loss of sporulation genes in several different lineages (staphylococci, streptococci, listeria, lactobacilli, ruminococci) in the course of their adaptation to the saprophytic lifestyle in a nutrient-rich environment. It argues that the systematics of *Firmicutes* is a rapidly developing area of research that benefits from the evolutionary approaches to the ever-increasing amount of genomic and phenotypic data and allows arranging these data into a common framework.

> Later the *Bacillus* filaments begin to prepare for spore formation. In their homogenous contents strongly refracting bodies appear. From each of these bodies develops an oblong or shortly cylindrical, strongly refracting, dark-rimmed spore.
>
> Ferdinand Cohn. 1876. Untersuchungen über Bacterien. IV. Beiträge zur Biologie der Bacillen. *Beiträge zur Biologie der Pflanzen*, vol 2, p 249–276. (Studies on the biology of the bacilli. In: Milestones in Microbiology: 1546 to 1940. Translated and edited by Thomas D. Brock. Prentice-Hall, Englewood Cliffs, NJ, 1961, p 49–56).

BACTERIAL SYSTEMATICS FROM GRAM STAIN TO 16S rRNA

The taxonomy of spore-forming Gram-positive bacteria has a long and colorful history. In 1872, 35 years after Christian Ehrenberg provided the initial description of *Vibrio subtilis* (and also *Vibrio bacillus*), Ferdinand Cohn assigned it to the genus *Bacillus* and family *Bacillaceae*, specifically noting the existence of heat-sensitive vegetative cells and heat-resistant endospores (see reference 1). Soon after that, Robert Koch identified *Bacillus anthracis* as the causative agent of anthrax in cattle and the endospores as a means of the propagation of this organism among its hosts. In subsequent studies, the ability to form endospores, the specific purple staining by crystal violet-iodine (Gram-positive staining, reflecting the presence of a thick peptidoglycan layer and the absence of an outer membrane), and the relatively low (typically less than 50%) molar fraction of guanine and cytosine in the genomic DNA have been used as diagnostic characteristics of the phylum *Firmicutes* (low-G+C Gram-positive bacteria).

Remarkably, neither of these traits proved to be a clear-cut predictor of the organism's membership in the *Firmicutes*. Many members of the phylum (lactic acid bacteria, listeria, staphylococci) do not form endospores, some *Firmicutes* stain Gram-variable or even Gram-negative, and some, like *Symbiobacterium thermophilum*, have the G+C content of >60%, which is more typical for the *Actinobacteria*.

Obviously, microorganisms can be classified by a variety of parameters, including the cell shape, staining

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pattern, spore formation, relation to oxygen, nutritional requirements, the ability to use CO_2 and fix nitrogen, salt tolerance, and, last but not least, pathogenicity. For many years, "numerical" taxonomy based on a combination of such parameters seemed the only way to impose some order onto the enormous diversity of microbial life. In many respects, it was successful, and the early descriptions of many bacterial species, genera, and families were later upheld by molecular techniques.

However, the deep-level systematics of bacteria required different approaches. The breakthrough came from the studies by Carl Woese and colleagues, who sought to base bacterial classification on the evolutionary history of the respective organisms and used the similarity of 16S rRNA sequences as a universal measure of the evolutionary proximity of the organisms (2–5). The 16S rRNA-based phylogenetic classification of bacteria has become universally accepted (see Table 1 for a list of resources) and proved so successful that it is sometimes hard to imagine that it is only 25 years old. In the case of Gram-positive bacteria, the 16S rRNA sequences were found to share conserved oligonucleotide signatures, lending molecular biology support for the Gram-staining results (3). Furthermore, 16S rRNA-based trees mostly agreed with phylogenetic trees based on other popular markers, such as ribosomal proteins, DNA gyrase subunit GyrB, RNA polymerase subunits, and others (6, 7). Subsequent more detailed analyses led to the recognition of the substantial differences between the low- and high-G+C Gram-positive bacteria, which had been assigned to two different phyla, the *Firmicutes* and the *Actinobacteria*, respectively. The recent genome-based studies confirmed the absence of a close relationship between the members of *Firmicutes* and *Actinobacteria*, justifying their separation into two different phyla. This chapter discusses the taxonomy of spore-forming *Firmicutes*, aiming to show that the importance of bacterial systematics goes beyond simply reflecting the current state of knowledge on the relatedness of various taxa within the phylum. Modern taxonomy strives to reflect the evolutionary history and serves as a guiding tool for further genome-sequencing projects and also for comparative genomics studies, which combine to provide a better understanding of bacterial physiology, including the sporulation processes in diverse members of the *Firmicutes*.

FIRMICUTES AS A SEPARATE EARLY-DIVERGING PHYLUM

In the absence of a reliable fossil record, any speculations on the timing of the divergence of the major bacterial phyla are bound to remain controversial. In addition, the bacterial phylogenetic tree appears to have

TABLE 1 Principal data sources on bacterial (*Firmicutes*) taxonomy

Data resource, reference, URL[a]	Comment
Taxonomy databases	
Approved Lists of Bacterial Names (103), http://www.ncbi.nlm.nih.gov/books/NBK814/	A list of validly published bacterial species names, last updated in 1989
Prokaryotic Nomenclature Up-to-date at the German Collection of Microorganisms and Cell Cultures (DSMZ), http://www.dsmz.de/bacterial-diversity/	An updated listing of validly published bacterial species names and nomenclature changes
Bergey's Manual of Systematic Bacteriology (32), http://www.bergeys.org/outlines.html	The official bacterial systematics from the Bergey's Trust
ITIS - Catalogue of Life (104), http://www.catalogueoflife.org/annual-checklist/	Integrated Taxonomic Information System, a partnership of North American government agencies
List of Prokaryotic Names with Standing in Nomenclature (42), http://www.bacterio.net/	A constantly updated listing of validly published species names, includes bacterial classification and *Candidatus* organisms
NCBI taxonomy database (41), http://www.ncbi.nlm.nih.gov/taxonomy	A hierarchical database of all organisms that have nucleotide sequences deposited in GenBank
The Taxonomic Outline of Bacteria and Archaea (31), http://www.taxonomicoutline.org/	Text-based bacterial taxonomy files (in PDF), updated in 2007
Taxonomic Outline of the Phylum *Firmicutes* (105), http://www.bergeys.org/outlines/bergeys_vol_3_outline_linked.pdf	Text-based *Firmicutes* taxonomy files (in PDF), updated in 2009
16S rRNA databases	
The SILVA database (106), http://www.arb-silva.de/	A constantly updated 16S rRNA-based Tree of Life
Greengenes (107, 108), http://greengenes.lbl.gov/	Includes an improved classification of uncultivated bacteria
Ribosomal Database Project (109), http://rdp.cme.msu.edu/	A constantly updated 16S rRNA-based Tree of Life

[a]The resources are listed in alphabetical order.

a starlike topology with all major phyla diverging at approximately the same time; only tentative groupings of some phyla ("superphyla") have been put forward (8–10). However, in the case of the *Firmicutes*, there is a general consensus that they had diverged from other bacterial phyla at a relatively early stage (8, 11, 12). The evolution of *Firmicutes* obviously included numerous events of lateral gene transfer to and from representatives of other phyla, which is why certain gene families are shared by *Firmicutes* with *Fusobacteria*, *Thermotogae*, and other groups (13, 14). Still, the core set of well-conserved informational genes and their protein products show much higher similarity within the members of the phylum than to any organisms from other phyla. The phylogenetic trees built from ribosomal proteins and/or RNA polymerase subunits are typically consistent with the 16S rRNA-based trees and show confident clustering of various *Firmicutes* members (9, 15–18). The unity of *Firmicutes* is also supported by other means of comparative genome analysis, including dinucleotide frequencies, codon usage, presence of simple sequence repeats, and distribution of insertion and deletions in highly conserved proteins (19–21), reviewed in references 11 and 22.

Much of the discussion of the *Firmicutes* evolution focuses on the absence of the outer membrane. Gupta and several other researchers argued that the presence of a single cytoplasmic membrane must be an ancient feature, unifying *Firmicutes* with *Actinobacteria*, *Mollicutes*, *Thermotogae*, and/or *Archaea*—and potentially *Chloroflexi*—into a single group *Monodermata* (11, 19, 23) or *Posibacteria* (24, 25) that was ancestral to Gram-negative bacteria (*Didermata* or *Negibacteria*). While severely criticized by others, see e.g., reference 26 and the discussion in reference 25, these ideas helped in highlighting the big—and still unresolved—questions of the early evolution of *Bacteria*. In any case, the distinct identity of the *Firmicutes* as a separate early diverging phylum is not being disputed by anyone. Even the highly controversial (and generally dismissed by other microbiologists) classification of bacteria proposed by Cavalier-Smith retained the *Firmicutes* as a separate class of *Teichobacteria*, based on the presence of teichoic acid in their cell walls (24).

THE EVOLVING SYSTEMATICS OF *FIRMICUTES*

While the existence of *Firmicutes* as a separate phylum is no longer a matter of contention, the systematics within the phylum is still very much in flux. Just in the past several years, one class (*Mollicutes*) has been removed from the *Firmicutes*, three new taxa have been elevated to the class level, a number of new taxa—at the genus, family, and order level—have been described, and some previously characterized species have been reassigned to new taxa.

As mentioned above, the classification of *Firmicutes*, as well as the assignment of new isolates to various taxa within this phylum, is based primarily on the 16S rRNA similarity patterns, the thickness of bacterial cell walls, and several additional traits. The reliance on the cell wall as a key diagnostic feature recently led to a noteworthy conflict between the taxonomic and phylogenetic approaches. The *Mollicutes* (mycoplasmas), which fall within the *Firmicutes* in most 16S rRNA and ribosomal protein-based trees (9, 15, 18, 27) and share with *Firmicutes* a number of common traits (28–30), had been previously considered a distinct class-level lineage within the *Firmicutes* (31). However, because *Mollicutes* lack the peptidoglycan cell wall, they have been reassigned to a separate phylum, the *Tenericutes* (32).

The removal of *Mollicutes* left the *Firmicutes* as a paraphyletic group with just two classes, *Bacilli* and *Clostridia*, both having a typical Gram-positive cell wall (31, 32). However, another group of *Firmicutes*, the family *Erysipelotrichaceae*, was found to contain an unusual type of peptidoglycan and share with *Mollicutes* a number of traits, which prompted its elevation to the class level (29, 32, 33). The genomes of two representatives, *Erysipelothrix rhusiopathiae* and *Eubacterium cylindroides*, have been sequenced (29), and two dozen other genomes are in the pipeline. Genome analysis is expected to shed light on the cellular physiology of these interesting bacteria, which include several spore-forming species that had been previously misassigned to the *Clostridium* genus (Table 2).

Two more lineages of *Firmicutes* have also been elevated to the class level, forming classes *Thermolithobacteria* and *Negativicutes* (32, 34, 35). The class *Thermolithobacteria* currently includes just two species, both appear to be asporogenous (34), and so far neither of them has been the subject of a genome-sequencing project. The last class, *Negativicutes*, unifies bacteria that stain Gram-negative (i.e., do not retain the Gram stain); in some carefully studied cases, they were seen surrounded by two membranes and had a thin cell wall (36). Nevertheless, based on 16S rRNA-, ribosomal proteins-, RpoB-, GyrB-, and DnaK-based trees, these bacteria are legitimate members of the *Firmicutes* phylum (18, 35, 37). Several representatives of this group have been shown to form endospores (36, 38, 39). The current classification

TABLE 2 Distribution of spore-forming bacteria among *Firmicutes*

Class, order[a]	Family[a]	Spore-forming members		
		Fraction[b]	Complete genomes[c]	Example (GenBank entry or reference)
Bacilli				
Bacillales	*Alicyclobacillaceae*	+++	2 (3)	*Kyrpidia tusciae* (CP002017)
	Bacillaceae	++	32 (73)	*Bacillus subtilis* (CP000922)
	Listeriaceae	–	– (28)	
	Paenibacillaceae	+++	6 (10)	*Paenibacillus polymyxa* (CP000154)
	Pasteuriaceae	+++	–	*Pasteuria penetrans* (110)
	Planococcaceae	+	1 (1)	*Solibacillus silvestris* (AP012157)
	Sporolactobacillaceae	++	–	*Sporolactobacillus inulinus* (AFVQ00000000)
	Staphylococcaceae	–	– (42)	
	Thermoactinomycetaceae	+++	–	*Desmospora activa* (111)
	Other	+	– (3)	*Tuberibacillus calidus* (112)
Lactobacillales	*Aerococcaceae*	–	– (1)	
	Carnobacteriaceae	–	–	
	Enterococcaceae	–	– (10)	
	Lactobacillaceae	–	– (44)	
	Leuconostocaceae	–	– (10)	
	Streptococcaceae	–	– (99)	
Clostridia				
Clostridiales	*Caldicoprobacteraceae*	+++	–	*Caldicoprobacter oshimai* (113)
	Catabacteriaceae	–	–	
	Christensenellaceae	–	–	
	Clostridiaceae	+++	25 (43)	*Clostridium botulinum* (CP000727)
	Defluviitaleaceae	+++	–	*Defluviitalea saccharophila* (114)
	Eubacteriaceae	–	– (9)	
	Gracilibacteraceae	–	–	
	Heliobacteriaceae	++	1 (1)	*Heliobacterium modesticaldum* (CP000930)
	Lachnospiraceae	++	– (7)	*Anaerostipes butyraticus* (115)
	Oscillospiraceae	–	– (1)	
	Peptococcaceae	++	11 (15)	*Desulfitobacterium hafniense* (CP001336)
	Peptostreptococcaceae	++	1 (11)	*Clostridium difficile* (AM180355)
	Ruminococcaceae	+	– (9)	*Sporobacter termitidis* (116)
	Syntrophomonadaceae	+	– (2)	*Pelospora glutarica* (117)
	Other	+	3 (7)	*Symbiobacterium thermophilum* (AP006840)
Halanaerobiales	*Halanaerobiaceae*	–	–	
	Halobacteroidaceae	–	– (4)	
Natranaerobiales	*Natranaerobiaceae*	–	– (1)	
Thermoanaero-bacterales	*Thermoanaerobacteraceae*	++	11 (13)	*Carboxydothermus hydrogeniformans* (CP000141)
	Thermodesulfobiaceae	–	– (2)	
	Other	+	2 (13)	*Mahella australiensis* (CP002360)
Erysipelotrichia				
Erysipelotrichales	*Erysipelotrichaceae*	+	– (2)	*Clostridium ramosum*
Negativicutes [d]				
Selenomonadales	*Acidaminococcaceae*	–	– (2)	
	Veillonellaceae	+	– (5)	*Pelosinus fermentans* (57, 58)
Thermolithobacteria				
Thermolithobacterales	*Thermolithobacteraceae*	–	–	

[a]Taxonomy is according to the List of Prokaryotic Names with Standing in Nomenclature (42) and the NCBI Taxonomy database (41); see Table 1 for the URLs.

[b]The distribution of sporeformers among the experimentally characterized members of the respective family is indicated as follows: +++, all (or nearly all) characterized members of the family produce spores; ++, a significant fraction of species are sporeformers; +, the family includes some sporeformers; –, no known sporeformers in the family.

[c]The number of spore-forming species with completely sequenced genomes in the respective family (according to the RefSeq database [56] as of November 1, 2012); the total number of completely sequenced genomes is given in parentheses.

[d]See reference 18 for a discussion on whether the order *Selenomonadales* deserves to be placed in the separate class *Negativicutes* as opposed to the class *Clostridia*.

of *Negativicutes* includes a single order *Selenomonadales* with two families, *Acidaminococcaceae* and *Veillonellaceae*, with sporeformers found only in the latter one (Table 2). It has been recently argued that elevation of Gram-negative *Firmicutes* to a separate class was not justified and that they should be left as a separate order within the class *Clostridia*, consistent with the 16S rRNA- and protein-based phylogenetic trees (18). In any case, this interesting group of *Firmicutes* is being intensively studied, and its systematics is likely to change in the future. Here, we just refer to these bacteria as members of the *Selenomonadales*.

Although the families *Bacillaceae* and *Clostridiaceae* were established and described in detail many years ago, orders *Bacillales* and *Clostridiales* were created only in 1953 by André-Romain Prévot, and the classes *Bacilli* and *Clostridia* were codified only recently to accommodate the rapidly growing number of newly described Gram-positive bacteria. Indeed, more than half of the families listed in Table 2 have been described only in the past 5 to 10 years. In other instances, selected genera have been elevated to the family level after molecular analysis showed that they were only distantly related to other genera in the same family.

It should be noted that, although bacterial taxonomy may seem to be in a constant flux, it is generally stable at the (intermediate) levels of family and order; many families of *Bacilli* and *Clostridia* that were described in the early 20th century are still recognized as such. Genera are somewhat less stable, because the description of new species often reveals new groupings and leads to the subdivision of a single genus into two or three new genera. This inevitably leads to the name change, sometimes affecting well-known and often-used organisms, such as *Bacillus* (now *Lysinibacillus*) *sphaericus*, *Bacillus* (now *Geobacillus*) *stearothermophilus*, and many others (Table 3). That said, *Clostridium difficile* still retains its name, even though molecular data revealed that it—along with several related *Clostridium* spp.—clearly falls outside the family *Clostridiaceae* and probably belongs in the family *Peptostreptococcaceae* (40). This has led to a recent proposal to rename it *Peptoclostridium difficile* and assign new names to 77 other former *Clostridium* species (18). Fortunately, such resources as the NCBI Taxonomy database (41) and the List of Prokaryotic Names with Standing in Nomenclature (42) keep track of the name changes and allow searches using the old names.

SPORE-FORMING AND ASPOROGENOUS *FIRMICUTES*

The ability to form spores depends on a conserved set of at least 60 genes, mutations of which interrupt the sporulation process at various steps and decrease the fraction of sporeformers by several orders of magnitude or even render the cells completely asporogenous (43–47). Accordingly, the ability to form spores is easily lost, and many lineages contain both spore-forming and non-spore-forming members. Some other lineages do not include any spore-forming members, suggesting that the ability to form spores was either lost very early in the history of that lineage or was absent from its ancestors (see below). As

TABLE 3 Recent renaming of some well-known sporeformers

Old name	New name (GenBank genome entry)[a]
Bacilli	
Bacillus acidocaldarius	*Alicyclobacillus acidocaldarius* (CP001727)
Bacillus brevis	*Brevibacillus brevis* (AP008955)
Bacillus globisporus	*Sporosarcina globispora*
Bacillus haloalkaliphilus	*Alkalibacillus haloalkaliphilus* (AKIF00000000)
Bacillus pantothenticus	*Virgibacillus pantothenticus*
Bacillus polymyxa	*Paenibacillus polymyxa* (CP000154)
Bacillus sphaericus	*Lysinibacillus sphaericus* (CP000817)
Bacillus stearothermophilus	*Geobacillus stearothermophilus*
Bacillus tusciae	*Kyrpidia tusciae* (CP002017)
Clostridia	
Clostridium fervidum	*Caloramator fervidus*
Clostridium lentocellum	*Cellulosilyticum lentocellum* (CP002582)
Clostridium thermoaceticum	*Moorella thermoacetica* (CP000232)
Clostridium thermohydrosulfuricum	*Thermoanaerobacter thermohydrosulfuricus*
Clostridium thermosaccharolyticum	*Thermoanaerobacterium thermosaccharolyticum* (CP002171)
Desulfotomaculum orientis	*Desulfosporosinus orientis* (CP003108)
Thermoanaerobacter tengcongensis	*Caldanaerobacter subterraneus* (AE008691)
Thermoanaerobium brockii	*Thermoanaerobacter brockii* (CP002466)

[a]GenBank accession number of the genome sequence, if available.

an example, the current classification subdivides the class *Bacilli* into two orders, *Bacillales* and *Lactobacillales*, which include, respectively, nine and six families (Table 2). There are no (known) spore-forming representatives within *Lactobacillales* and in two families of *Bacillales*, *Listeriaceae*, and *Staphylococcaceae*; the remaining seven families of *Bacillales* contain both spore-forming and apparently asporogenous members. There are four recognized orders in the class *Clostridia*; two of them (*Clostridiales* and *Thermoanaerobacterales*) include sporeformers, whereas the other two (*Halanaerobiales* and *Natranaerobiales*) do not (Table 2).

It must be noted that the information on whether a particular organism forms spores is somewhat biased; the presence of spores in the culture positively identifies the bacterium as a sporeformer, whereas the absence of the visible spores is not sufficient to label the organism as asporogenous. Indeed, the absence of spores in a studied sample could be due to the specific isolation and cultivation conditions; the organism's inability to form spores cannot be ascertained without a specific concerted effort to detect spore formation under a variety of growth conditions. Thus, even when light or electron microscopy and/or a heat resistance test indicate the absence of spores in the culture, there remains a distinct possibility that proper conditions for the organism's sporulation have not yet been found.

As an example, the thermophilic, facultatively chemolithoautotrophic anaerobe *Thermincola ferriacetica* has been observed to forms spores (48). In contrast, its close relative *Thermincola carboxydiphila* has not been seen to do that (49), but there has been no special effort to detect spore formation in its culture (E.A. Bonch-Osmolovskaya, personal communication). The third member of the genus, *Thermincola potens*, had its genome sequenced without microbiological characterization of the organism (50). Thus, there is no easy way to predict whether *T. potens* is a sporeformer, even though its genome appears to encode all essential sporulation proteins (44). Hopefully, the perspective of using *T. potens* in microbial fuel cells (51, 52) would lead to a better microbiological description of this interesting organism.

Spore formation is a particularly interesting trait for the members of the *Selenomonadales*. Since these bacteria stain Gram negative, many newly characterized members of this group have only been checked for sporulation with the use of light microscopy. Nevertheless, the reports on the inability of many members of the *Selenomonadales* to form spores appear to be correct and are supported by the available genomic data. Sporulation has been observed in some representatives of *Veillonellaceae*, such as *Sporomusa sphaeroides* and *Acetonema longum* (36, 38, 39), but seems to be restricted to just a handful of genera (Table 2) that belong to a separate branch of the phylogenetic tree (18, 53). Further, even within that branch there are reported non-spore-forming species, such as, for example, *Sporomusa paucivorans* (54). In such cases, a recent loss of the ability to sporulate seems very likely.

Summing up, a number of taxa within the phylum *Firmicutes* contain both spore-forming and asporogenous members. The apparently independent loss of the ability to form endospores in distinct lineages of this phylum suggests that, despite providing a clear evolutionary advantage when it comes to surviving environmental challenges, sporulation comes with its own costs. Accordingly, adaptation of many members of the phylum (lactic acid bacteria, staphylococci, and others) to their specific (e.g., nutrient-rich) ecological niches apparently included a loss of their ability to form spores.

COVERAGE OF THE *FIRMICUTES* DIVERSITY BY GENOME-SEQUENCING PROJECTS

Phylogenetic Diversity

The medical, environmental, and industrial importance of many Gram-positive bacteria fueled a sustained effort in genome sequencing of numerous members of the *Firmicutes* phylum. By the end of October 2012, almost five hundred complete genomes of various *Firmicutes* had been available in the public databases (see http://www.ebi.ac.uk/genomes/bacteria.html or ftp://ftp.ncbi.nih.gov/genomes/Bacteria/), there were also several hundred partially sequenced genomes and over 3,000 genome sequencing projects (see http://www.genomesonline.org/) (55, 56). The majority of the sequenced genomes came from well-characterized genera, such as *Bacillus*, *Clostridium*, *Staphylococcus*, and *Streptococcus*, with multiple complete genomes of various strains of *Bacillus subtilis* and the human pathogens *Bacillus anthracis*, *Clostridium botulinum*, *Clostridium difficile*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* and the insect pathogen *Bacillus thuringiensis*. However, owing largely to the efforts of the Genomic Encyclopedia of Bacteria and Archaea (GEBA) project (45), there is now a fairly detailed genomic coverage of the *Firmicutes* diversity, with complete genomes available for representatives of all (currently recognized) orders —and most families—of *Bacilli* and *Clostridia*, as well as several representatives of *Negativicutes* and *Erysipelotrichia* (see http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html).

TABLE 4 Genome sequencing of extremophilic sporeformers

Organism name	Growth conditions	GenBank accession number, reference
Acidophiles		
Alicyclobacillus acidocaldarius	pH_{opt} 3.5(pH 2–6)	CP001727 (118, 119)
Sulfobacillus acidophilus	pH_{opt} 1.8(pH 1.6–2.3)	CP002901 (83, 120); CP003179 (121, 122)
Alkaliphiles		
Alkaliphilus metalliredigens	pH_{opt} 9.5(pH 7.5–11)	CP000724 (123)
Bacillus halodurans	pH_{opt} 9.5(pH 7.0–11)	BA000004 (70, 124)
Bacillus pseudofirmus	pH_{opt} 8.5–10.6(pH 7.5–11.4)	CP001878 (125, 126)
Halophiles		
Oceanobacillus iheyensis	3.6 M NaCl(0%–21%)	BA000028 (66, 67)
Virgibacillus halodenitrificans	4.2 M NaCl(2%–25%)	ALEF00000000 (127, 128)
Thermophiles		
Alicyclobacillus acidocaldarius	T_{opt} 65°C(45–70°C)	CP001727 (118, 119)
Thermoanaerobacter mathranii	T_{opt} 70–75°C(50–75°C)	CP002032 (129)
Psychrophiles		
Bacillus weihenstephanensis	T = 4°C(4–35°C)	CP000903 (130)
UV-resistant strains		
Bacillus pumilus SAFR-032	UV_{254} <1 kJ/m^2	CP000813 (131, 132)

So far, complete genomes of sporeformers have come exclusively from the representatives of *Bacilli* and *Clostridia*. As mentioned above, most members of *Erysipelotrichia* are asporogenous. However, *Clostridium ramosum*, *Clostridium innocuum*, and *Clostridium spiroforme*, recently reassigned to this class, are sporeformers, whose draft genome sequences are already available. Among *Selenomonadales*, complete genomes are currently available for several non-spore-forming representatives, such as *Acidaminococcus intestini*, *Acidaminococcus fermentans*, *Megasphaera elsdenii*, *Selenomonas ruminantium*, and *Veillonella parvula*, and just one sporeformer, *Sporomusa ovata* (133). However, draft genomes of six metal-reducing strains of the sporeformer *Pelosinus fermentans* have been sequenced and assembled into 65, 76, 98, 134, 844, and 887 contigs, respectively (57, 58). In addition, a genome-sequencing project of another sporeformer, *Acetonema longum*, has been brought to the level of 296 contigs. There is also a draft genome of the non-spore-forming *Thermosinus carboxydivorans* (59). Remarkably, each of these unfinished genomes encodes orthologs of more than 60 key sporulation proteins of *B. subtilis*, confirming the overall unity of the sporulation machinery in all *Firmicutes*. Our recent study found that the set of sporulation proteins encoded in the unfinished genomes of *A. longum*, *P. fermentans*, and *T. carboxydivorans* was essentially the same as that encoded in most clostridial genomes, lending credence to the suggestion that *Selenomonadales* are just very unusual members of the *Clostridia* (18).

Obviously, for comparative purposes, it would be advantageous to have at least two representative genomes from each major lineage (e.g., at the genus level), but the existing genome coverage has already allowed some meaningful comparisons, see e.g., references 17, 44, 46, 60–62.

Ecological Diversity

The breadth of the genomic coverage of the *Firmicutes* is also reflected in the variety of ecological niches inhabited by already sampled organisms. The ability of spores to survive environmental challenges, such as heat, desiccation, presence of organic solvents and oxidizing agents, and UV irradiation, as well as predation by protozoa (63–65), helped spore-forming *Firmicutes* colonize a wide variety of diverse habitats. Sporeformers inhabit most aquatic and terrestrial habitats, both aerobic and anaerobic, and have been found in a variety of environments, including deep in the ocean (e.g., *Oceanobacillus iheyensis* and *Geobacillus kaustophilus* [66–68]) and in the Earth's crust ("*Candidatus*[1] Desulforudis audaxviator" [69]).

In the past several years, complete genome sequences have become available for a number of Gram-positive extremophiles (Table 4), including acidophiles, alkaliphiles, thermophiles, psychrophiles, and halophiles. Again, the existing genome coverage, while far from exhaustive, has already allowed some interesting comparative analyses (67, 70). Still, genome sequences remain to be determined from many extremophilic sporeformers, such as, for example, *Psychrobacillus* spp. that can grow even at –2°C (71, 72).

It is important to note that the ability to grow at extreme conditions is not exclusive for sporeformers; there are many extremophiles in other phyla, as well as non-

[1]The *Candidatus* name is used for incompletely described organisms, including those that have not been cultivated (and deposited in internationally recognized culture collections).

spore-forming extremophiles among *Firmicutes*. For example, no spores were observed in the culture of the obligately halophilic and alkaliphilic (growth at >3 M NaCl and pH >8.3) thermophilic bacterium *Natranaerobius thermophilus*, a member of *Clostridia* isolated from a soda lake in Egypt (73). Although its genome encodes a nearly complete set of sporulation genes, it lacks both *dpaB* and *etfA* genes that code for two subunits of clostridial dihydrodipicolinate reductase, an essential sporulation enzyme (44, 74, 75). The extreme thermophiles *Ammonifex degensii* and *Caldicellulosiruptor* spp. are also nonsporeformers (76–80).

Model Organisms

Aside from the popular model organism *B. subtilis*, spore-forming *Firmicutes* are probably most famous for the diseases that they cause, which include anthrax, food poisoning, infectious diarrhea, enterocolitis, gas gangrene, tetanus, and various kinds of bacteremia. However, their importance is not limited to pathogenicity. Such organisms as *B. thuringiensis* are being actively used for pest control, and there are now more than two dozen completely sequenced genomes of various strains of *B. thuringiensis* that differ in their insecticidal activity. Clostridia are being studied for their potential use in production of biofuel (*C. acetobutylicum*) and/or wood processing (*C. cellulolyticum*, *C. cellulovorans*, *C. clariflavum*, *C. thermocellum*), and genome sequencing is increasingly being used to analyze the encoded hydrolases and their ability to degrade cellulose, lignin, and other components of plant cell walls (81).

In physiological terms, spore-forming *Firmicutes* include both autotrophs and heterotrophs, many of which have been used as model organisms for biochemical and biophysical studies and have completely sequenced genomes. Chemolithoautotrophs include a variety of hydrogen- or formate-oxidizing bacteria that grow by reducing sulfur, sulfate, or nitrate (69, 82). Other strains grow by oxidizing minerals, including ferrous iron (83). A number of sporeformers are capable of utilizing carbon monoxide. As its name suggests, *Carboxydothermus hydrogenoformans* produces molecular hydrogen (84), whereas *Clostridium ljungdahlii* can use CO/H_2 and CO_2/H_2 mixtures (85). The family *Heliobacteriaceae* includes phototrophic members that use anaerobic anoxygenic photosynthesis as a source of energy; they are also able to fix nitrogen (86–88). The photosynthetic reaction centers of *Heliobacillus mobilis* and *Heliobacterium modesticaldum* represent some of the most primitive photosynthetic systems and are being studied to understand the mechanisms and evolution of photosynthesis. *Bacillus methanolicus* is a sporeformer that can utilize methanol as its sole carbon and energy source; unfinished genome sequences of two of its strains (7 and 12 contigs, respectively) were released in 2012 (89). Further studies are bound to find new unexpected applications of sporeformers, such as the above-mentioned use of *T. potens* in microbial fuel cells (51).

GENOMICS OF SPORULATION

Diagnostic Sporulation Genes

Comparative analyses of diverse *Firmicutes* genomes identified a core set of sporulation genes that are conserved in (nearly) all sporeformers (43, 44, 46, 47, 90) and could be considered a "sporulation genomic signature" (91). Incidentally, most of these genes are also essential for sporulation: the respective mutations affect sporulation in *B. subtilis*, *C. acetobutylicum,* and/or other model organisms, resulting in the decrease of spore count by two orders of magnitude or more. Unfortunately, the attempts to identify tell-tale sporulation genes proved unsuccessful; there was not a single gene that would be present in all sporeformers and absent in all asporogens (44).

One of the best indicators of the spore-forming ability is Spo0A, the master regulator of sporulation. Spo0A is a transcriptional regulator that combines the two-component receiver domain with a specific type of the helix-turn-helix DNA-binding domain, which so far has been seen only among the members of *Firmicutes* (92). However, Spo0A is also encoded in the genomes of several unequivocally non-spore-forming organisms, such as *Caldicellulosiruptor* spp. (77–79), *Exiguobacterium* spp. (93), *Macrococcus caseolyticus* (94), and many others (44) (see Table 5). Still, Spo0A can be used as a molecular marker for evaluating the abundance of sporeformers in natural environments (134).

In addition to *spo0A*, other apparently sporulation-specific genes are occasionally found in the genomes of nonsporeformers (Table 5) and can be seen even outside of the phylum *Firmicutes* (43, 44, 62). On the other hand, owing to the phenomenon of nonorthologous gene displacement (i.e., the ability of unrelated or distantly related proteins to perform the same function), some essential sporulation genes can be missing in certain genomes. A good example is the ability of the electron transfer flavoprotein EtfA to catalyze the oxidation of dihydrodipicolinate into dipicolinate, a universal component of the developing spore (75). This activity underlies replacement of the *dpaA* and *dpaB* genes by *etfA* in such spore-forming clostridia as

TABLE 5 Distribution of sporulation genes in some non-spore-forming bacteria[a]

Taxonomy: class, order	Sporulation genes											
	spo0A	*spmA*	*spo0M*	*spoIIM*	*spoIIIAA*	*spoIVA*	*spoIVFA*	*spoVAC*	*spoVG*	*spoVR*	*spoVS*	*sspF*
Class *Bacilli*												
Order *Bacillales*												
Bacillus selenitireducens	+	–	–	–	–	–	–	–	+	–	+	–
Macrococcus caseolyticus	+	–	(–)	–	–	–	–	–	+	–	–	–
Exiguobacterium sibiricum	+	–	+	v	–	–	–	–	+	–	+	–
Class *Clostridia*												
Order *Clostridiales*												
Clostridiales genomosp. BVAB3	+	–	–	–	–	–	–	–	–	–	–	–
Eubacterium rectale	+	–	–	–	+	+	+	+	+	–	–	+
Eubacterium eligens	+	–	–	+	+	+	–	+	+	–	–	+
Roseburia hominis	+	–	–	–	–	–	–	+	+	–	–	+
Oscillibacter valericigenes	+	+	–	–	+	+	+	+	+	–	–	+
Ethanoligenens harbinense	+	+	–	–	+	+	+	+	+	+	+	+
Ruminococcus albus 7	+	–	–	–	+	+	+	+	+	–	+	+
Syntrophomonas wolfei	+	+	–	+	+	+	+	+	–	–	+	+
Thermaerobacter marianensis	+	+	–	+	+	+	+	+	+	+	+	+
Order *Halanaerobiales*												
Acetohalobium arabaticum	+	+	–	+	+	+	+	+	–	–	+	+
Halanaerobium praevalens	+	–	–	–	–	–	–	–	+	–	+	–
Halothermothrix orenii	+	+	–	+	+	+	+	+	+	–	+	+
Order *Thermoanaerobacterales*												
Ammonifex degensii KC4	+	+	–	+	+	+	+	+	–	+	+	+
Caldicellulosiruptor saccharolyticus	+	+	–	+	+	+	+	+	+	–	+	+
Other phyla												
Actinobacteria	–	–	+	+	–	–	–	–	–	–	+	–
Cyanobacteria	–	+	+	+	+	–	–	–	–	+	–	–
Proteobacteria	–	+	+	+	+	–	–	–	+	+	–	–
Chloroflexi	–	–	+	+	+	–	–	–	–	+	+	–
Spirochetes	–	–	–	–	–	–	–	–	+	–	–	–
Thermotogae	–	–	–	–	–	–	–	–	–	–	+	–
Euryarchaeota	–	–	+	+	–	–	–	–	+	+	–	–

[a]Based on the data from references 44 and 62.

C. acetobutylicum, *C. botulinum*, and *C. perfringens* (44, 75). A nonorthologous gene displacement of *spoIIQ* or *spoIVFA* has been proposed to explain the absence of these genes in clostridia (44).

What Is the Minimal Genome Size of a Sporeformer?

A recent genome comparison of spore-forming and asporogenous *Firmicutes* revealed a certain degree of correlation between the ability of the bacterium to form spores and its genome size. Most *Firmicutes* whose completely sequenced genomes have sizes of more than 3 million base pairs (Mbp) were found to be sporeformers (44). The few exceptions among *Clostridia* included *Oscillibacter valericigenes* (4.7 Mbp), *Eubacterium limosum*, and *Eubacterium rectale* (4.5 and 3.6 Mbp, respectively), *Butyrivibrio proteoclasticus* (3.8 Mbp), and certain representatives of *Enterococcus*, *Lactobacillus*, *Roseburia*, and *Ruminococcus* genera. Among *Bacilli*, the only exceptions were *Bacillus selenitireducens* (3.6 Mbp), *Exiguobacterium* spp. (3.0 Mbp), and *Listeria* spp. (3.0 Mbp). This correlation also showed up in the observation that all cultured *Firmicutes* with genome sizes of less than 2.3 Mbp were asporogenous; these included the majority of lactobacilli and streptococci (44). It was concluded that, at least among free-living *Firmicutes*, sporulation was a property of relatively gene-rich bacteria.

Among the free-living representatives of classes *Bacilli* and *Clostridia*, the lower boundary of genome size for sporeformers was estimated at 2.8 Mbp in *Anoxybacillus flavithermus* (17) and 2.3 Mbp in *Thermoanaerobacter mathranii*, respectively (44).

However, since the ability to form spores appears to depend on a just a few dozen genes (43, 44, 46), it is quite likely that there exist uncharacterized free-living spore-forming *Firmicutes* with much smaller genome sizes. Such organisms are likely to be found in relatively stable environments, such as the open sea or the Earth's crust, that have not yet been sufficiently sampled. Besides, organisms with relatively small genomes are likely to have limited metabolic capabilities and therefore can be expected to be fastidious and harder to cultivate.

Indeed, there already is an example of sporeformers whose genome sizes are much smaller than 2.3 Mbp. These organisms are referred to as unculturable segmented filamentous bacteria and are closely related to the genus *Clostridium*, forming a proposed genus "*Candidatus* Arthromitus" in the *Clostridiaceae* family (95, 96). Over the years, these bacteria have been seen attached to the intestinal walls of many animals, including mice, rats, cats, dogs, and chickens; a dedicated study using light microscopy expanded the range of hosts of "*Ca*. Arthromitus" to include human, monkeys, domestic fowl, toad, and carp (97, 98). Although "*Ca*. Arthromitus" spp. have not yet been cultivated outside of the mammalian hosts, they have been shown to form spores (99), and the ability of the spores to survive treatment with 3% chloroform has been used to obtain a (nearly) pure culture of these bacteria, suitable for genome sequencing (96).

The 1.5- to 1.6-Mbp genome sequences of three strains of "*Ca*. Arthromitus" spp. proved to be much smaller than those of any free-living sporeformers, owing largely to the apparent loss of genes responsible for amino acid biosynthesis, carbohydrate and nucleotide metabolism, and energy conservation (96, 100, 101). At the same time, "*Ca*. Arthromitus" spp. encoded a relatively large number of sporulation proteins, most likely comprising a nearly minimal core set of essential sporulation genes (44), see references 96 and 101. We should not exclude the possibility of eventually finding sporeformers with even smaller genimes—and probably even more dependent upon the host for the supply of essential nutrients.

Evolution of Sporulation

The presence of a conserved set of sporulation genes that confers the ability to form endospores in two different, early diverging branches of *Firmicutes*, *Bacilli*, and *Clostridia*, not to mention the *Selenomonadales* (*Negativicutes)*, strongly suggests that it was already present in their common ancestor. An alternative explanation, horizontal transfer of numerous (at least sixty, probably many more) sporulation genes from one branch to another after their separation, sounds extremely unlikely. However, the assumption that sporulation ability is an ancestral feature would indicate that the various asporogenous lineages within the *Firmicutes* phylum (see Table 2) lost (most of) their sporulation genes relatively late in their evolution, after the separation from the closely related spore-forming lineages. For saprophytic and fastidious bacteria (listeria, staphylococci, streptococci, etc.), the loss of sporulation genes could have been part of a systemic genome compaction in the course of their adaptation to the relatively nutrient-rich ecological niches. Indeed, representatives of these lineages not only have significantly smaller genomes than their spore-forming relatives, but they also encode fewer biosynthetic pathways (e.g., references 61 and 102). This genome compaction, accompanied by the loss of metabolic genes, is particularly evident in the case of *Erysipelothrix rhusiopathiae*, which lacks the genes coding for the enzymes of the tricarboxylic acid

cycle, fatty acid biosynthesis, synthesis of biotin, riboflavin, pantothenate, thiamine, and folate and a number of amino acids (29). Given this scale of genome compaction, the complete loss of sporulation genes in *Erysipelothrix* is hardly surprising.

Several years ago, a study of an *Anoxybacillus flavithermus* strain isolated from a super-saturated silica solution revealed a genome sequence that was one-third shorter than that of *B. subtilis* and, accordingly, encoded 33% fewer proteins (17). This finding prompted an examination of gene conservation among the 20 bacillar, 5 clostridial, and 6 mollicute genomes sequenced by that time, which led to a somewhat paradoxical conclusion that the common ancestor of all *Firmicutes* might have encoded just 1,318 protein families (17). Taking into account paralogy and "orphan" reading frames, that number would still correspond to fewer than 2,000 genes. This relatively small genome was then dramatically expanded during the evolution of *Bacillaceae* lineage and somewhat contracted in the *Anoxybacillus/Geobacillus* branch (17). Such reconstructions are necessarily tentative and depend strongly on the available set of complete genomes. Still, they suggest that the genome of the common ancestor of *Firmicutes* could have been reasonably close to the smallest genomes of modern free-living spore formers, such as *Thermoanaerobacter mathranii* and *A. flavithermus*.

CONCLUDING REMARKS

Spore-forming members of the phylum *Firmicutes* are extremely diverse in their biochemical, physiological, and ecological properties and range from obligate parasites to free-living phototrophs and chemolithotrophs. Many sporeformers attract wide interest, either as model organisms, or because of the diseases they cause, or because of their potential use in biotechnology, bioremediation, or insect control. Despite their diversity, all sporeformers share a common heritage and encode very similar sets of sporulation genes that they likely inherited from a common ancestor of all *Firmicutes*. The ability to form spores has been lost numerous times in a variety of lineages of the *Firmicutes*, usually in the course of adaptation to life in nutrient-rich conditions. This indicates that the ability to survive environmental challenges by forming endospores comes with certain strings attached, which could be a reason why it is not found anywhere outside the *Firmicutes* phylum.

While this chapter was in preparation, the worldwide genome-sequencing efforts brought us complete and draft genomes of many new members of the *Firmicutes*, including some spore-forming species (55, 56, 133). These sequence data keep feeding the new science of phylogenomics, which uses comparative genomics data to reconstruct bacterial evolution and aims at a better understanding of the entire phenomenon of life.

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