In Silico Analytical Tools for Phylogenetic and Functional Bacterial Genomics

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Abstract Microbial significance in human lives has been gaining importance due to their biotechnological applications and ability to cause diseases. The use of antibiotics to kill them has proved counterproductive. Bacterial resistance to antibiotics has caused huge economic losses. Many bacteria have turned highly drug resistant due to modifications in their genetic reservoirs. It has been recognized that bacteria had another mechanism to circumvent the impact of antibiotics. Bacteria causing infectious diseases form biofilm at high cell density. Biofilm protects bacteria from even extremely high dosages of antibiotics. Under all these conditions, the most important aspect to initiate treatment is to diagnose the organism responsible for the disease. Bacterial identification through the *rrs* gene sequence has been the most prevalent and effective approach. The trouble arises in two main situations: (1) high similarity among gene sequences and (2) the presence of multiple copies of *rrs* gene within a genome. An obvious solution is to employ other highly conserved genes (housekeeping genes), which is uneconomical in terms of time and money. However, a few studies have revealed the presence of latent features within *rrs*. A set of genomic tools allowed identification of organisms up to the species level from their previous status of genus level identity. The most interesting aspect is that the strategy can be extended to all genes from all kinds of organisms.

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1 Introduction

Human interest in microbes has been growing steadily as their applications in our daily life have gained importance (Kalia 2015b; Moroeanu et al. 2015). The role of bacteria in causing diseases has always been studied with the intention of finding mechanisms to kill them. The discovery of antibiotics proved extremely helpful in reducing human misery. Since antibiotics are targeted to kill bacteria (Alipiah et al. 2015), they respond to this stress and undergo genetic changes, which are expressed as functional changes. The immediate impact of these changes has been the evolution of drug resistance in bacteria (Saxena et al. 2014). It was also realized that without undergoing any genetic change, bacteria under certain conditions may show enhanced antibiotic resistance. This acquired antibiotic resistance was attributed to the biofilm formed by bacteria by quorum sensing (QS), a phenomenon which is controlled by cell density. Bacteria under the regulation of QS express virulent metabolic behavior. Under this new regime, the approach is to inhibit QS and prevent bacteria from acquiring resistance to antibiotics (Gui et al. 2014; Kalia 2013a, 2014a, b; Prakasham et al. 2014). Hence, QS system is turning out to be a novel drug target (Kalia and Purohit 2011; Agarwala et al. 2014; Shang et al. 2014; Hema et al. 2015; Kaur et al. 2015; Koul et al. 2015b; Arya and Princy 2016). In all these scenarios, the need is to identify bacteria and provide a rapid diagnosis, which will permit the treatment to proceed.

Initially, phenotypic and biochemical characteristics were used as the basis for their identification and classification. The developments in molecular biology, genomic, and bioinformatics have changed the pace of research in these organisms. The turning point in the new era of genomics came into effect primarily because of the insights provided by Prof. Carl R. Woese (Kalia 2013b; Prakash et al. 2013; Mahale et al. 2014). Microbiologists around the world have used the tools developed in the last three to four decades to identify bacteria: PCR-ribotyping, microarray analysis, restriction endonuclease (RE) digestion, amplified fragment length polymorphism, multi-locus sequence analysis, DNA hybridization, isotope distribution analysis, molecular connectivity, etc. (Sharma et al. 2008; Kapley and Purohit 2009; Nguyen et al. 2013; Prakash et al. 2014; Wang et al. 2014; Yu et al. 2014, 2015; Meza-Lucas et al. 2016; Yagnik et al. 2016).

2 Bacillus

Bacillus is a versatile organism, which has been exploited for a large number of biotechnological applications. This genus has encompassed such a large number of diverse organisms that may be equated with *Pseudomonas*, as the "dumping" ground for gram-positive organisms (Porwal et al. 2009). This genus represents a lot of phenotypic and genotypic heterogeneity, such that an unambiguous identification up to species level has been a tough task (Porwal et al. 2009). Members of

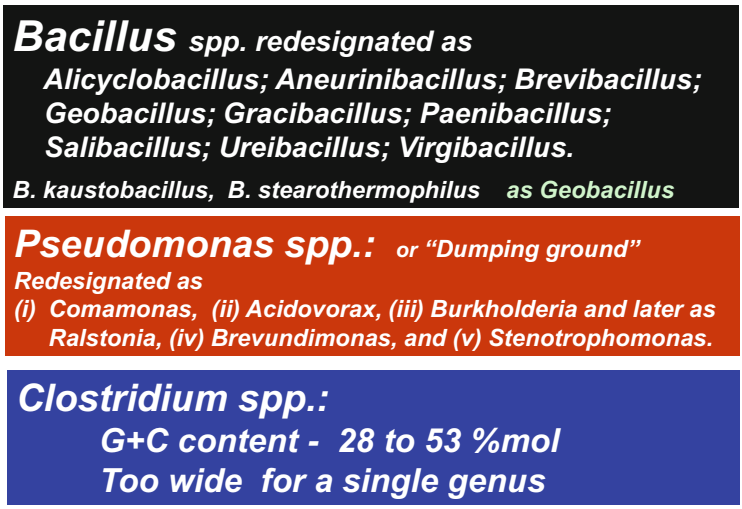

Fig. 1 Reorganization of bacterial systematics: *Bacillus*, *Pseudomonas*, and *Clostridium* (Porwal et al. 2009; Kalia et al. 2011a, b; Bhushan et al. 2013)

Bacillus subtilis group, *B. cereus* group, *B. licheniformis*, and *B. sphaericus* are some of the most notorious trouble spots among *Bacillus* spp. High genomic similarity between *B. subtilis* and *B. halodurans* is recorded for G+C content, genome size, and the characteristics of their ABC transporter genes, ATPases, etc. Information presented by the complete genome of *B. halodurans* show similarity for the enzymes transposases and recombinases, with those recorded among *Anabaena*, *Clostridium*, *Enterococcus*, *Lactococcus*, *Rhodobacter*, *Staphylococcus*, and *Yersinia* species. It clearly hints that *Bacillus* needs further segregation into new genera: *Aneurinibacillus*, *Ureibacillus*, *Virgibacillus*, *Brevibacillus*, and *Paenibacillus*. In fact, *Bacillus stearothermophilus*, *B. thermoleovorans*, *B. kaustophilus*, and *B. thermoglucosidasius* are categorized as *Geobacillus*, whereas *B. pasteurii*, *B. globisporus*, and *B. psychrophilus* are now known as *Sporosarcina* spp. (Fig. 1). The members of *Bacillus marinus* are presently classified as *Marinibacillus marinus* (Yoon et al. 2001).

3 *Clostridium*

The biotechnological importance of *Clostridium* has made researchers to monitor this organism with curiosity and precision. The bacteria is benign on one hand as it can produce solvents, enzymes, biofuels like butanol, ethanol, hydrogen, etc. and is extremely dangerous on the other hand, with the ability to produce deadly toxins (Carere et al. 2008; Bhushan et al. 2015). The heterogeneity of *Clostridium* has been recorded in phenotypic, biochemical, and genotypic characteristics. For quite

some time, the issue of accommodating organisms varying in GC content from as low as 24 mol% (*Clostridium perfringens*) to as high as 58 mol% (*Clostridium barkeri*) did not appear justified (Fig. 1) (Kalia et al. 2011a).

4 *Pseudomonas*

Just like *Clostridium* and *Bacillus*, another equally important organism is *Pseudomonas*. In spite of these developments, there are still quite a few bacteria which were otherwise clubbed together and needed reclassification. Almost all organisms which were difficult to categorize were labeled as *Pseudomonas*. It was comprised of phenotypically and biochemically highly diverse organisms and was named the "dumping ground" (Fig. 1). They have a versatile metabolic ability to infect and degrade almost everything and no doubt are among the most widely studied pathogens and biodegraders (Bhushan et al. 2013). *Pseudomonas* has been subjected to repeated taxonomic revisions (Lalucat et al. 2006; Peix et al. 2009). Combined use of housekeeping genes such as*gyrB*, *rpoB*, *rpoD*, *rec*A, *atp*D, and *car*A and the classic gene—*rrs*—was found to be effective in distinguishing different species of *Pseudomonas*: *P. flavescens*, *P. mendocina*, *P. resinovorans*, *P. fluorescens*, *P. chlororaphis*, *P. aeruginosa*, *P. syringae*, *P. putida*, *P. stutzeri*, etc. (Spiers et al. 2000; Hilario et al. 2004; Aremu and Babalola 2015).

5 *Stenotrophomonas*

Another highly versatile organism is *Stenotrophomonas* spp. The phylogenetic diversity of *Stenotrophomonas* spp. is quite interesting as its members were initially grouped under *Pseudomonas* and *Xanthomonas*. Presently, eight recognized *Stenotrophomonas* spp. exist: *S. maltophilia*, *S. nitritireducens*, *S. acidominiphilia*, *S. rhizophila*, *S. koreensis*, *S. terrae*, *S. humi*, and *S. chelatiphaga*. *Stenotrophomonas dokdonensis* has been transferred to *Pseudoxanthomonas* as *P. dokdonensis*. As far as the functional abilities of *Stenotrophomonas* species are concerned, they are able to treat aromatic compounds either individually or in combination with *Bacillus*, *Pseudomonas*, *Flavimonas*, and *Morganella* spp. Ecological and metabolic (genetic and functional) diversity of *S. maltophilia* implies high taxonomic heterogeneity (Anzai et al. 2000; Peix et al. 2007; Tourkya et al. 2009; Verma et al. 2010, 2011; Aremu and Babalola 2015).

6 *Streptococcus*

The genus *Streptococcus* has a big number of species with important clinical relevance. Severe and acute diseases are known to be caused by species such as *S. agalactiae*, *S. pneumoniae,* and *S. pyogenes*. Different analytic methods enable identification to a limited extent and are laborious to apply. The genetic variability among different *Streptococcus* groups is quite low, and distinguishing them is a tough task (Lal et al. 2011; Kalia et al. 2016).

7 *Helicobacter*

Another group of organisms, which have great economic importance and are a cause of worry for health departments, belong to the genus *Helicobacter* (Puri et al. 2016). These organisms cause many diseases in human beings. Among the different species of *Helicobacter*, the following show high genetic variability: *H. cinaedi*, *H. pylori*, *H. felis, H. bilis*, and Candidatus *H. heilmannii*. Previously, *Helicobacter* spp. were categorized as *Campylobacter* sp. (Goodwin et al. 1989). Since *H. pylori* is responsible for 50 % of the infections caused by different relatives of *Helicobacter* (Suerbaum and Michetti 2002), it happens to be the most extensively studied species with 450 sequenced genomes. Biochemical assays, including urease test, are cheap but may not be very accurate. Molecular techniques like PCR and MLSA have also not proved to be highly precise (Puri et al. 2016).

8 The Novel Approach to Exploit Hidden Talents of *rrs*

With constant research efforts over the last three decades, *rrs* gene sequencing technique has been simplified to such an extent that almost all research laboratories around the globe have adopted it as routine assay. The RDP database has become a rich referral center, to which the newly sequenced *rrs* gene is subjected and the best match is used for identifying the organism. It must, however, be realized that RDP database can identify the new organism only against what is already known and deposited. The database cannot classify a gene sequence which has not been seen by it so far. It therefore requires a novel overture, wherein we need to first define the taxonomic and phylogenetic limits of each known species and key out the disruptions in the evolutionary scale. In a serial publication of works undertaken in this guidance, extensive genomic analyses were performed to look for any potential characteristics of *rrs* genes, which have not been elucidated so far. In the following text, a few case studies will be described to elucidate the approach, its validity, and significance (Fig. 2) (Kalia 2015a).

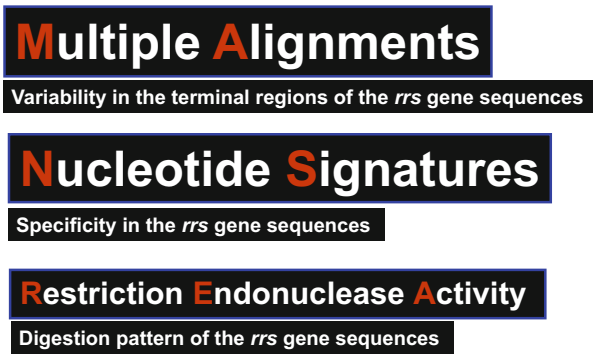

Fig. 2 Novel molecular techniques to explore the microbial taxonomy and phylogeny (Porwal et al. 2009; Kalia et al. 2011a; Lal et al. 2011; Bhushan et al. 2013; Puri et al. 2016)

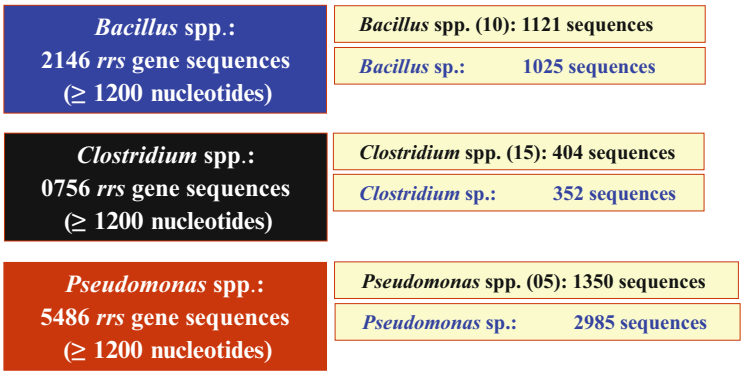

Fig. 3 Number of *rrs* gene sequences of *Bacillus*, *Clostridium*, and *Pseudomonas* used for developing phylogenetic framework—(1) identified up to species level and (2) those identified only up to genus level (Porwal et al. 2009; Kalia et al. 2011a; Bhushan et al. 2013)

9 Bacillus

9.1 Phylogenetic Framework

The first step was to define the phylogenetic boundaries of a species within a given genus. *Bacillus* was the first genus to be studied to evaluate the potential of this approach. Out of the available information in the database (at that time), 1121 *rrs* gene sequences of 10 *Bacillus* species were taken into consideration: *B. thuringiensis, B. anthracis, B. pumilus, B. cereus, B. subtilis, B. megaterium, B. sphaericus, B. clausii, B. halodurans,* and *B. licheniformis* (36–211 strains) (Figs. 3 and 4) (Porwal et al. 2009). Phylogenetic trees developed on the basis of

Organism	No. of *rrs* gene sequences	No. of aligned groups
Bacillus anthracis	153	04
B. cereus	211	04
B. thuringiensis	108	10
B. subtilis	271	26
B. licheniformis	131	10
B. pumilus	83	12
B. megaterium	47	08
B. sphaericus	42	06
B. clausii	39	06
B. halodurans	36	04
Bacillus sp.		
Total	1121	90

Fig. 4 Segregation of *rrs* gene sequences of *Bacillus* species into multiple sequence alignment groups on the basis of variability in the terminal regions (Adapted from Open Access article: Porwal et al. 2009. doi:10.1371/journal.pone.0004438)

1121 allowed their segregation into 89 clusters. From each cluster, the outermost and innermost *rrs* gene sequence was considered as representative of the limits of the species. In case, there were a large number of sequences in a cluster; additional 1–2 sequences were also taken into account. In all cases, the type strain of the species was also used to develop the phylogenetic framework. On the basis of these criteria, a comprehensive framework consisting of 34 *rrs* sequences representing 10 different *Bacillus* species were established (Fig. 5). With this genomic tool in hand, 305 *Bacillus* strains which were identified only up to genus level could be reclassified as members of these 10 known species (Fig. 6) (Porwal et al. 2009). On the basis of this genomic study, it was proposed to segregate the strains of *B. subtilis* into two species/subspecies. It was revealed in the literature that on the biochemical basis, *B. subtilis* can be divided into subspecies—*subtilis* and *spizizenii* (Nakamura et al. 1999). The study was limited only to 10 species out of around 189 species which are reported to be known today. This indicates that there is a lot of scope to extend this work.

10 Unique Signatures

In order to validate the authenticity of the segregation of *rrs* sequences of strains which could be classified among known *Bacillus* species, 20–30 nucleotide long unique signatures were identified among the 10 known species using MEME program (http://meme.nbcr.net/meme/meme.html). The uniqueness of these

Organism	Accession numbers of Reference *rrs* gene sequence(s)
Bacillus thuringiensis	DQ286308(T)[a], DQ286339, DQ328630, AE017355, DQ286329
B. anthracis[b]	AB190218, AE017334, AE017225
B. cereus[b]	DQ372919, DQ289988
B. subtilis	AB042061(T), DQ420172, AY995569, DQ504376, AY583216, AY881635, AY631853
B. licheniformis	AB039328(T), CP000002, AF234855
B. pumilus	AY260861(T), AY876289, DQ523500
B. megaterium	AJ717381(T), AY373358, AY505510, AY373360
B. sphaericus	AJ310084(T), DQ286309
B. clausii	X76440(T), AB201793, AY960116
B. halodurans	AY423275(T), AY856452
Total	34 strains

Fig. 5 Phylogenetic framework sequences of *rrs* genes of ten *Bacillus* species. [a]Type strain. [b]For *B. cereus* group as a whole, only one type strain was used (Adapted from Open Access article: Porwal et al. 2009. doi:10.1371/journal.pone.0004438)

Bacillus sp.	No.
B. cereus	75
B. thuringiensis	02
B. anthracis	01
B. sphaericus	23
B. licheniformis	21
B. halodurans	07
B. megaterium	69
B. pumilus	32
B. subtilis	44
B. clausii	31
Total 305 / 1025	

Pseudomonas sp.	No.
P. aeruginosa	219
P. fluorescens	463
P. putida	347
P. stutzeri	197
P. syringae	141
Total 1367 / 2985	

Clostridium sp.	No.
15 species	179
Total 179 / 352	

Fig. 6 Number of *rrs* gene sequences of different organisms identified up to species level with the help of genomic frame work (Data on *Bacillus* has been adapted from Open Access article: Porwal et al. 2009. doi:10.1371/journal.pone.0004438) (Porwal et al. 2009; Kalia et al. 2011a; Bhushan et al. 2013)

signature sequences was verified by carrying out a blast search against all the sequences available at NCBI (Fig. 7) (Porwal et al. 2009). The motifs (signature sequences) were reported to be unique to a species, if these were absent from other species. Two to five 29–30 nucleotide long unique signatures were detected for

Bacillus spp. and Nucleotide Signatures	
Bacillus cereus	AAAGTGGAATTCCATGTGTAGCGGTGAAAT
B. thuringiensis	ATAACATTTTGAACTGCATGGTTCGAAATT
B. clausii	AATCCCATAAAGCCATTCTCAGTTCGGATT
B. halodurans	ATAATAAAAAGAACTGCATGGTTCTTTTTT
B. pumilus	AAGGTTTAGCCAATCCCACAAATCTGTTCT
B. megaterium	ATGATTGAAAGATGGTTTCGGCTATCACTT
B. sphaericus	TAAAACTCTGTTGTAAGGGAAGAACAAGTA

Fig. 7 Representative unique nucleotide signatures for *rrs* gene sequences of different *Bacillus* species. No unique signature was detectable for *B. anthracis*, *B. subtilis*, and *B. licheniformis* (Data adapted from Open Access article: Porwal et al. 2009. doi:10.1371/journal.pone.0004438)

Bacillus cereus, *B. thuringiensis*, *B. clausii*, *B. pumilus*, *B. megaterium*, *B. sphaericus*, and *B. halodurans*. In the cases of *B. anthracis*, *B. licheniformis*, and *B. subtilis*, unique signatures were not detectable.

A very interesting observation was made among signatures detected in *rrs* gene sequences of organisms which were identified only as *Bacillus* sp. Their nucleotide signatures indicated that either these organisms belong to those *Bacillus* spp. which have not been used in this study (Porwal et al. 2009) or they represent some other genus/genera. A few of these signatures did show a close resemblance to organisms belonging to *Virgibacillus, Geobacillus, Jeotgalibacillus, Brevibacillus, Marinibacillus, Paenibacillus,* and *Pontibacillus* (Porwal et al. 2009). A survey of published works revealed that some of these organisms (still classified as *Bacillus* sp.) had been reclassified and belong to *Virgibacillus, Geobacillus, Jeotgalibacillus, Brevibacillus, Marinibacillus, Paenibacillus,* and *Pontibacillus* (Heyndrickx et al. 1999; Nazina et al. 2001). It reflected on the strength of the study, which with the help of *in silico* analysis alone provided evidences that these *Bacillus* spp. needed segregation as new genera or species.

11 Restriction Endonuclease Digestion Analysis

Another unique feature to further support the segregation of organisms on the basis of *rrs* gene was the identification of RE, which elucidated a unique digestion pattern. Here the best part was the number of fragments, their size (nucleotides), and the order in which they occur within the gene.

Fourteen type II REs (Table 3) were used: (1) four base pair cutters (*AluI*, *HaeIII*, *DpnII*, *BfaI*, *Tru9I*, and *RsaI*), (2) six base pair cutters (*EcoRI*, *BamHI*, *NruI*, *SmaI*, *HindIII*, *PstI*, and *SacI*), and (3) eight base pair cutter (*NotI*) (rebase.neb.com/rebase/rebase.html). These REs were selected due to the occurrence of highly specific cleavage sites. It was realized that out of these 14 REs, only four base

***Rsa* I**

Bacillus anthracis									406	♦		355	♦	146					
B. thuringiensis									406	♦		355	♦	146					
B. cereus									406	♦		355	♦	146					
B. halodurans								171	♦	235	♦	357	♦	146					
B. megaterium			16	♦	11	♦		406		♦		356	♦	146	♦				
B. clausii	♦		19	♦	11	♦		406		♦		356	♦	146					
B. licheniformis	♦		19	♦	11	♦		406		♦		501			♦				
B. sphaericus	♦		18	♦	11	♦		405		♦		496							
B. subtilis	♦		19	♦	11	♦		407		♦		502			♦				
B. pumilus						♦		409		♦		502							

***Hae*III**

Bacillus anthracis					22	♦		565	♦		34	♦	456						
B. thuringiensis				♦	22	♦		568	♦		34	♦	457	♦					
B. cereus				♦	22	♦		565	♦		34	♦	457						
B. halodurans			78	♦	22	♦			598			♦	459						
B. megaterium			78	♦	22	♦			578			♦	459						
B. clausii	♦		78	♦	22	♦		85	♦	216	♦	264	♦						
B. licheniformis				♦	22	♦			598			♦	457	♦					
B. sphaericus	♦		78	♦	22	♦			596			♦	452	♦					
B. subtilis				♦	22	♦			600			♦	457						
B. pumilus				♦	22	♦			599			♦	458	♦					

Fig. 8 *In silico* restriction endonuclease digestion of *rrs* gene sequences of different *Bacillus* species with *RsaI* and *HaeIII*. Values represent the fragment size (nucleotides). The *filled symbol* represents the RE action site (Data adapted from Open Access article: Porwal et al. 2009. doi:10.1371/journal.pone.0004438)

pair cutter could be exploited as these REs generated 5–9 fragments with sizes, which can be easily distinguished even under experimental conditions (Figs. 8 and 9). RE-*RsaI* digestion of *rrs* of different *Bacillus* spp. resulted in 2–6 fragments ranging in size from 11 to 502 nucleotides. *B. cereus* group members gave similar digestion patterns and were indistinguishable among them. *B. halodurans* and *B. pumilus* were easily distinguished from others based on their unique RE digestion patterns (Fig. 8). *B. clausii* and *B. sphaericus* could be identified as distinct on digestion with RE *HaeIII* (Fig. 8). *In silico* digestion of *rrs* of *B. megaterium*, and *B. pumilus*, gave a unique pattern with RE Tru9I (Fig. 9). The presence of two sets of unique digestion patterns in *rrs* sequences belonging to *B. subtilis* with RE *AluI* (Fig. 9) provided a strong evidence of the potential segregation of this group into two subspecies or as separate species. This observation was made in the phylogenetic framework analysis described above. It may be remarked that certain species segregate together in one RE can be distinguished by analyzing the digestion patterns with another RE.

***Tru*9I**	***Bacillus anthracis***	**527 ♦ 270 ♦ 8 ♦ 86 ♦ 134 ♦ 44**
	B. thuringiensis	**527 ♦ 270 ♦ 8♦ 86 ♦ 134 ♦ 44♦**
	B. cereus	**♦ 527 ♦ 270 ♦ 8♦ 85 ♦ 134 ♦ 44**
	B. halodurans	**270 ♦ 8♦ 86 ♦ 136**
	B. megaterium	**280 ♦ 86 ♦ 137 ♦**
	B. clausii	**270 ♦ 8♦ 86 ♦ 134 ♦**
	B. licheniformis	**♦ 278 ♦ 86 ♦ 134 ♦**
	B. sphaericus	**♦ 278 ♦ 86 ♦ 136**
	B. subtilis	**♦ 278 ♦ 89 ♦ 134 ♦ 181♦**
	B. pumilus	**331 ♦ 86 ♦ 134 ♦**

***Alu*1**	***Bacillus anthracis***	**174 ♦ 224 ♦ 599 ♦ 185 ♦ 21♦ 58**
	B. thuringiensis	**174 ♦ 225 ♦ 599 ♦ 186 ♦ 21♦ 58**
	B. cereus	**174 ♦ 224♦ 581♦ 42 ♦ 186 ♦ 21♦ 58**
	B. halodurans	**173 ♦ 824**
	B. megaterium	**86 ♦ 88 ♦ 615 ♦ 209 ♦ 265 ♦ 123**
	B. clausii	**85 ♦ 88 ♦ 186♦ 218♦ 419♦**
	B. licheniformis	**136♦ 33 ♦ 822 ♦ 265**
	B. sphaericus	**175 ♦ 615 ♦ 209 ♦ 265 ♦**
	B. subtilis	**♦ 173 ♦ 186♦ 431♦ 207♦ 265 ♦**
		♦ 164 ♦ 42 ♦ 779 ♦ 374
	B. pumilus	**85 ♦ 88 ♦ 185♦ 430♦ 207♦ 265 ♦**

Fig. 9 *In silico* restriction endonuclease digestion of *rrs* gene sequences of different *Bacillus* species with *Tru9I* and *AluI*. Values represent the fragment size (nucleotides). The *filled symbol* represents the RE action site (Data adapted from Open Access article: Porwal et al. 2009. doi:10.1371/journal.pone.0004438)

12 *Clostridium*

The approach described above for developing genomic tools for phylogenetic analysis were extended to *rrs* gene sequences of *Clostridium* (Kalia et al. 2011a). Here 756 rrs sequences of 110 *Clostridium* species were taken into consideration. *Clostridium botulinum rrs* gene sequences were segregated into four groups. Out of these four groups of *C. botulinum*, 10 *rrs* sequences were selected to represent 128 sequences (Fig. 3). By drawing phylogenetic trees of 15 different *Clostridium* species, 56 *rrs* gene sequences were selected for creating a phylogenetic framework. It defined the phylogenetic limits of the *C. acetobutylicum, C. butyricum, C. beijerinckii, C. perfringens, C. botulinum, C. chauvoei, C. baratii, C. pasteurianum, C. colicanis, C. sardiniense, C. subterminale, C. novyi, C. sporogenes, C. kluyveri,* and *C. tetani*. With this genomic tool in hand, 356 *Clostridium* strains identified only up to genus level could be classified among these 15 known species (Kalia et al. 2011a). A confirmation of this initial reclassification was achieved through nucleotide signature analysis and unique RE digestion patterns. In this case also, REs—*HaeIII*, *AluI*, *RsaI*, *DpnII*, *Tru9I*, and *BfaI*—proved effective in providing relevant information. RE—AluI—was instrumental in

Clostridium sp.	Restriction Enzyme (AluI) digestion fragments (nucleotides, nts)
C. pasteurianum	■ 49 ■ 186 ■ 610 ■ 207 ■ 142 ■
C. pasteurianum	■ 12 ■ 49 ■ 186 ■ 610 ■ 207 ■ 142 ■
C. pasteurianum	■ 62 ■ 186 ■ 610 ■ 349 ■ 41 ■
C. pasteurianum	■ 61 ■ 186 ■ 610 ■
C. chauvoei	■ 61 ■ 186 ■ 610 ■ 206 ■ 57 ■
C. baratii	■ 186 ■ 609 ■ 207 ■ 57 ■ 109 ■
C. sardiniense	■ 186 ■ 604 ■ 206 ■ 57 ■ 109 ■
C. botulinum	■ 170 ■ 566 ■ 230 ■ 207 ■ 57 ■ 109 ■
C. botulinum	■ 566 ■ 228 ■ 205 ■ 57 ■ 109 ■
C .acetobutylicum	■ 797 ■
C. subterminale	■ 170 ■ 796 ■ 207 ■ 143 ■
C. sporogenes	■ 795 ■ 264 ■ 85 ■
C. botulinum	■ 170 ■ 795 ■ 265 ■ 85 ■
C. botulinum	■ 796 ■ 264 ■ 85 ■
C. butyricum	■ 170 ■ 797 ■ 264 ■ 109 ■
C. sporogenes	■ 170 ■ 796 ■ 264 ■ 85 ■
C. acetobutylicum	■ 170 ■ 185 ■ 609 ■ 266 ■
C. perfringens	■ 186 ■ 609 ■ 264 ■ 109 ■
C. perfringens	■ 186 ■ 609 ■ 264 ■
C. perfringens	■ 186 ■ 609 ■ 264 ■
C. colicanis	■ 186 ■ 607 ■ 264 ■ 109 ■
C. beijerinckii	■ 170 ■ 184 ■ 607 ■ 264 ■ 109 ■
C. butyricum	■ 170 ■ 186 ■ 611 ■ 264 ■
C. butyricum	■ 170 ■ 186 ■ 610 ■ 264 ■ 109 ■
C. beijerinckii	■ 186 ■ 608 ■ 264 ■ 109 ■
C. kluyveri	■ 186 ■ 609 ■ 351 ■
C. subterminale	■ 345 ■ 450 ■ 347 ■
C. tetani	■ 173 ■ 345 ■ 452 ■
C. novyi	■ 172 ■ 358 ■ 437 ■
C. botulinum	■ 358 ■ 437 ■

Fig. 10 *In silico* restriction endonuclease digestion of *rrs* gene sequences of different *Clostridium* species with *AluI*. Values represent the fragment size (nucleotides). The *filled symbol* represents the RE action site (Data adapted from Open Access article: Kalia et al. 2011a. doi:10.1186/1471-2-2164-12-18)

clearly segregating *C. chauvoei*, *C. acetobutylicum*, *C. kluyveri*, *C. perfringens*, *C. colicanis*, *C. pasteurianum*, and *C. subterminale* (Fig. 10) (Kalia et al. 2011a).

13 *Pseudomonas*, *Helicobacter*, and *Streptococcus*

Using approaches similar to those defined above for *Bacillus* and *Clostridium* spp., it was found that effective and meaningful phylogenetic and taxonomical information can be retrieved also in the cases of *Stenotrophomonas*, *Streptococcus*, *Pseudomonas*, and *Helicobacter* (Verma et al. 2010, 2011; Lal et al. 2011; Bhushan et al. 2013; Puri et al. 2016).

14 Functional Genomics

In addition to using the genomic tools primarily for bacterial identification, it was realized that these can be extended to derive meaningful information in other genes as well. In attempts to inhibit the virulent behavior of bacteria causing infectious diseases, a search for organisms producing bioactive molecules to act as antibacterial was conducted. Since most infectious diseases are caused by organisms which produce biofilm through QS system, anti-QS molecule producers were targeted. Two enzymes—acyl-homoserine lactone acylase and acyl-homoserine lactone lactonase—have been shown to inhibit QS. Phylogenetic and functional

genomic analyses of the genes responsible for the production of these enzymes were carried out. Unique signatures and RE digestion patterns enabled to establish the phenomenon of horizontal gene transfer as well (Kalia et al. 2011b). The unique signatures were proposed as candidates for designing primers for detecting such organisms in unknown samples. On the basis of this functional genomic analysis, three organisms were traced, which possessed genes for both the AHL inhibitory enzymes (Kalia et al. 2011b; Kalia 2014a). The RE digestion of AHL-lactonase with *Tru9I, RsaI*, and *DpnII* could validate the phylogenetic segregation of the organisms based on *rrs* gene (Huma et al. 2011). Diversity analysis of *Citrobacter* species isolated from diverse niches was carried out to check their abilities to degrade aromatic compounds. Nine strains having genes, which coded for aromatic ring-hydroxylating dioxygenases, were analyzed using a diversity of REs—*DpnII*, *RsaI*, and *HaeIII*. Unique signature analysis in combination with RE showed that genomic similarity in a few specific strains supported their closeness in metabolic functions as well (Selvakumaran et al. 2011). Functional genomics of *Stenotrophomonas* diversity in effluent treatment plants was established with precision using RE digestion strategy. This enabled the development of a consortium to be used for bioremediation (Verma et al. 2010, 2011).

More recently, the genomic tool—RE digestion pattern—has been extensively used for identification of organisms, which are economically highly significant for health departments. The primary objective was to find novel markers to be used for diagnostic purposes (Arasu et al. 2015). The use of functional genes was necessitated by the multiple copies of *rrs* genes in different species of *Clostridium, Vibrio, Yersinia, Staphylococcus, Streptococcus*, and *Lactobacillus* (Kalia et al. 2015, 2016; Kalia and Kumar 2015; Kekre et al. 2015; Koul et al. 2015a; Koul and Kalia 2016; Kumar et al. 2016).

15 Opinion

Phylogenetic analysis based on gene sequences is a very handy and effective tool. The gene most widely used for bacterial identification and overall taxonomical purposes is *rrs*. Although it is a widely employed technique, it leads into trouble quite frequently. Often, the most obvious choice is to employ other highly conserved genes (HKGs). It, however, implies higher inputs of time and money. Invariably, additional 7–8 HKGs are required to resolve the issue. To circumvent the efforts needed for identifying bacteria using a single gene—*rrs*—a fresh round of studies were conducted, to develop genomic tools: phylogenetic framework, signatures, and RE digestion patterns. Once again, these tools ran into trouble in case of organisms which possessed multiple copies of *rrs*. The potential solution seems to lie in the genes common to all the species within a genus. Unique gene-RE digestion pattern allowed identification of novel biomarkers. It thus can be envisaged that the use of specific REs-gene combinations can be used for all kinds of phylogenetic and functional genomic analysis.

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