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REVIEW

Polyphasic approach of bacterial classification – An overview of recent advances

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Abstract Classification of microorganisms on the basis of traditional microbiological methods (morphological, physiological and biochemical) creates a blurred image about their taxonomic status and thus needs further clarification. It should be based on a more pragmatic approach of deploying a number of methods for the complete characterization of microbes. Hence, the methods now employed for bacterial systematics include, the complete 16S rRNA gene sequencing and its comparative analysis by phylogenetic trees, DNA-DNA hybridization studies with related organisms, analyses of molecular markers and signature pattern(s), biochemical assays, physiological and morphological tests. Collectively these genotypic, chemotaxonomic and phenotypic methods for determining taxonomic position of microbes constitute what is known as the 'polyphasic approach' for bacterial systematics. This approach is currently the most popular choice for classifying bacteria and several microbes, which were previously placed under invalid taxa have now been resolved into new genera and species. This has been possible owing to rapid development in molecular biological techniques, automation of DNA sequencing coupled with advances in bioinformatic tools and access to sequence databases. Several DNA-based typing methods are known; these provide information for delineating bacteria into different genera and species and have the potential to resolve differences among the strains of a species. Therefore, newly isolated strains must be classified on the basis of the polyphasic approach. Also previously classified organisms, as and when required, can be reclassified on this ground in order to obtain information about their accurate position in the microbial world. Thus, current techniques enable microbiologists to decipher the natural phylogenetic relationships between microbes.

Key words Polyphasic · Bacterial classification

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Introduction

Microbial taxonomy or microbial systematics deals with the classification, identification and nomenclature of microorganisms[1-5]. The terms Taxonomy and Systematics have been often used interchangeably but the two differ in their meaning. While Taxonomy is the theory and practice of classifying organisms[6], Systematics refers to the study of diversity of organisms and all relationships among them including their evolutionary relatedness (phylogeny) and all possible biological interactions[6,7].

The techniques prevalent a few decades back were not sufficient to provide a complete draft on which bacterial

taxonomy could be based. Most descriptions at that time were based on certain traits such as shape, color, size, staining properties, motility, host-range, pathogenicity and assimilation of a few carbon sources[8,9]. There was however a need for a more comprehensive approach to furnish convincing information to derive bacterial lineages. Scientists have now sought newer tools to study the evolutionary relationship among prokaryotes with much more precision than ever before.

The first attempt of microbial classification based on single-stranded DNA was made by Schildkraut *et al.* in 1961[10]. This was a major breakthrough in the world of microbial classification paving the way towards development of the polyphasic system of classification in its present form. Polyphasic approach is a recent trend in microbial taxonomy, which provides natural and authentic system of classification of microbes. The term coined by Colwell in 1970, refers to the integration of genotypic, chemotypic and phenotypic information of a microbe in order to perform reliable grouping of the organism[11]. This field is rapidly expanding and therefore it is imperative to update readers with the recent technical advances. This review attempts to present the practices currently employed to classify microbes and newer developments in the field of microbial taxonomy and phylogeny.

Concept of Bacterial Classification: Polyphasic Approach

The purpose of a classification system is to construct groups that are homogeneous in the sense that they consist of descendents of the nearest common ancestor[6]. Species, being the fundamental unit of biological classification, is critical for describing, understanding and comparing biological diversity from different ecological niches. In the most accepted 'concept of Biological species', species is defined as a group of individuals capable of interbreeding with each other to produce fertile progeny but are incapable of doing so with members of other species[12]. This concept however is not applicable to asexually reproducing organisms like bacteria.

A more appropriate approach also known as polyphasic approach is used to distinguish bacterial species based on morphological and biochemical data supplemented with information obtained from molecular techniques. Advances in polyphasic approach for bacterial classification such as 16S rRNA gene sequencing[8] and molecular fingerprinting techniques[13] integrated with other molecular markers have become important tools in microbial systematics. A number of criteria that include genotypic, chemotypic and phenotypic features used for polyphasic characterization of bacteria (Fig. 1) are discussed below.

I. Genotypic Methods

Several definitions for bacterial species were proposed earlier but with the advent of genotypic methods like 16S rRNA gene sequencing and DNA-DNA hybridization, the concept of bacterial species has been refined.

A 'bacterial species' is defined as a group of strains sharing 70% or more DNA-DNA relatedness with 5°C or less ΔTm value (Tm is the melting temperature of the hybrid) among members of the group, provided that all the phenotypic and chemotaxonomic features agree with the above definition. This is further corroborated from data on 16S rRNA gene sequence analysis wherein bacterial strains showing more than 3% sequence divergence are considered to be the members of different species[14,1,2,4,8].

a. DNA-Based Typing Methods

Advances in DNA-based molecular techniques have revolutionized the bacterial identification system and the field of bacterial taxonomy. These techniques include Restriction Fragment Length Polymorphism (RFLP), Plasmid profiling, Ribotyping, Amplified Ribosomal DNA Restriction Analysis (ARDRA), Pulse Field Gel Electrophoresis (PFGE)[15] and Randomly Amplified Polymorphic DNA (RAPD)[16].

Classically subtyping was performed by biochemical (biotyping), serological (serotyping), phage and antibacteriocin typing methods. However, DNA-based molecular typing methods have become more popular and widely acceptable due to their reproducibility, simplicity, high discriminatory power and thus help to avoid strain duplications. A number of modern techniques are proficient to classify bacteria upto strain level[3,4] and with every year passing by the list is growing exhaustive.

A few of these are described below:

PFGE uses *in situ* lysis of bacterial whole-cells in agarose plugs. The digested bacterial plugs are positioned in agarose gels and subjected to electrophoresis in an apparatus in which polarity of current changes at specified intervals of time. The method can resolve very large DNA fragments (10 to 800 kb in size)[17] and the DNA fragments are visualized on the gel following staining. This technique is used in taxonomical studies to distinguish bacterial isolates as separate strains wherein similar PFGE profile means strain relatedness[15].

RFLP and plasmid DNA profiling are preliminary typing methods that generate restriction profile of the DNA and of the plasmid respectively. It is based on random distribution of restriction sites in the genome. The type of profile generated depends on the group of bacteria under consideration

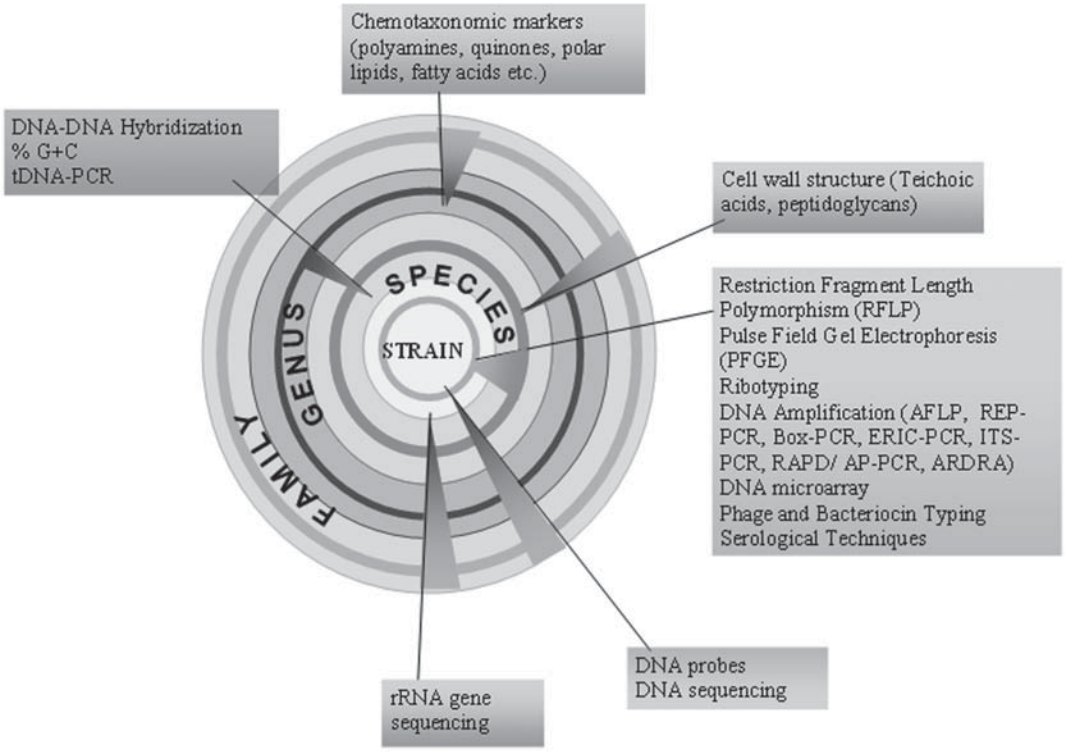


Fig. 1 Diagrammatic representation of techniques and markers used in modern polyphasic approach for resolving the bacterial hierarchy. For example, the chemotaxonomic markers determine the bacterial isolate upto genus whereas RFLP (restriction fragment length polymorphism) and PFGE (pulse field gel electrophoresis) can resolve the same upto strains level.

as well as on the type of restriction enzymes used. These methods however have their own shortcomings. RFLP generates complex profiles, which are difficult to compare, while plasmid profile may not be consistent as it is difficult for bacteria to maintain plasmids over several generations[13]. The profile can be simplified by probing it with gene specific probes. RFLP is thus often used in conjunction with Southern blotting.

Other methods like ribotyping and ARDRA are derivatives of RFLP. In ribotyping, rRNA, rDNA or gene specific oligonucleotides are used as probes against enzyme restricted DNA. Due to multiple copy number of 16S rRNA gene (1-14 depending up on the group of bacteria), a complex profile is obtained[18]. Presently, riboprinters are used to automate the method and read the profile. ARDRA employs digestion of amplified ribosomal DNA with different restriction enzymes and a profile is obtained using the combination of these patterns. This can be applied to screen large number of isolates simultaneously[19].

AFLP (Amplified Fragment Length Polymorphism) is another offshoot of RFLP in which specific adaptors are ligated to enzyme restricted DNA which is subsequently amplified using primers from the adaptor and restriction site-specific sequences. Here also the profile generated on the gel is used to discern species.

The Random Amplified Polymorphic DNA (RAPD) also referred to as arbitrary Primed PCR (AP-PCR) is another techinque in which short primer sequences (octa- to decamer) randomly anneal to genomic DNA and initiate amplification. If the primer anneal in proper orientation such that the distance between the annealing sites is a few kb apart then a PCR product is yielded. This results in a number of amplified fragments which when resolved on the gel generates a strain specific profile [20]. This technique has recently been used by Czekajlo *et al.* in 2006 to identify several strains of *Pseudomonas aeruginosa* [21].

Rep-PCR is another DNA amplification based technique for resolution of bacteria upto strain level[22,23]. Rep-PCR genomic fingerprinting is based on the amplification of naturally occurring, highly conserved, and repetitive DNA sequences, which are present in multiple copies throughout the genomes of most bacteria[24]. So far three families of repetitive DNA sequences have been identified: a) the Repetitive Extragenic Palindromic (REP) elements, 35-40 bp in size, and contain a variable loop in their structure[25], b) Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences are 124-127 bp in size and have central, conserved palindromic structures[26] and c) BOX elements which are 154 bp in size and have conserved subunits named as boxA, boxB, and boxC[27]. Among all subunits only the boxA-l subunit se-

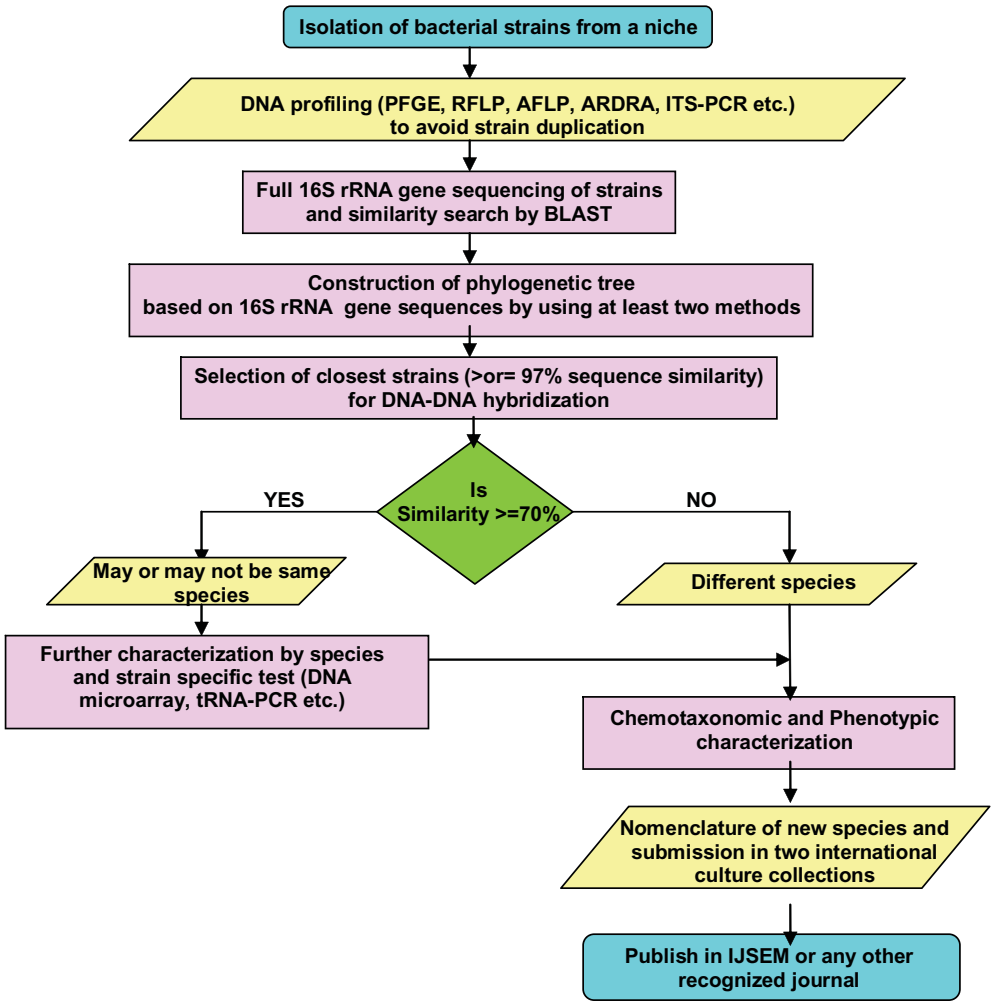


Fig. 2 Flow diagram depicting the step-by step procedure for taxonomical characterization of newly isolated strains followed by its deposition in culture collection centers and publication.

quences are highly conserved among bacteria. For typing purposes primers are designed to amplify DNA starting outward from the inverted repeats in REP and ERIC, while in case of BOX the direction is from boxA subunit [22]. By using these primers selective regions located between REP, ERIC or BOX elements can be amplified and corresponding method is called REP-PCR, ERIC-PCR and BOX-PCR genomic fingerprinting respectively. Collectively these three methods are referred to as rep-PCR genomic fingerprinting[28,22]. The amplified fragments can be resolved in a gel matrix and function as signature for specific bacterial strain.

Apart from these, tRNA-PCR and Intergenic Transcribed Spacer-PCR (ITS-PCR) can also be used for bacterial taxonomy. tRNA-PCR can also be used to amplify the spacers (interspersed region) between tRNA genes and distinguishes bacteria upto species level. While, ITS-PCR exploits the polymorphism within the intergenic transcribed spacer of 16S-23S rRNA and can be used for characterizing bacterial strains and hence, can serve as an important tool for phylogenetic analysis[29].

b. Inference of Phylogenetic Relationship by use of 16S rRNA Sequencing

In 1980s, studies concluded that phylogenetic comparison based on conserved part of genome was much stable than classification solely based on phenotypic traits and other features[8,31,9]. Hence the use of rRNA molecules was propagated for making phylogenetic comparisons[9,30]. All the three kinds of rRNA molecules i.e., 5S, 16S, 23S and spacers between these can be used for phylogenetic analyses but the small and large size of 5S rRNA (120 bp) and 23S rRNA (3300 bp) respectively have restricted their use. 16S rRNA gene (1650 bp) is the most commonly used marker that has revolutionized the field of microbial systematics[3,5,30]. Dubnau and coworkers in 1965 for the first time reported the conservation

in the 16S rRNA gene sequence in *Bacillus*[31]. Its widespread use in bacterial taxonomy started after the pioneering work of Woese in 1987[30]. 16S rRNA gene sequence is thus used for inferring the phylogenetic relationship among bacteria based on its universal distribution, highly conserved nature, fundamental role of ribosome in protein synthesis, no horizontal transfer and its rate of evolution which represents an appropriate level of variation between organisms[8,3,14]**.** The 16S rRNA molecule comprises of variable and conserved regions and universal primers for amplification of full 16S rRNA gene are usually chosen from conserved region while the variable region is used for comparative taxonomy. A list of universal primers frequently used in bacterial 16S rRNA gene sequencing is given in Table1. The 16S rRNA gene sequence is deposited in databases such as Ribosomal Database Project II (http://rdp.cme.msu.edu/) and GenBank (http://www.ncbi.nlm.nih.gov/). Sequences of related species for comparative phylogenetic analysis can also be retrieved from these databases. There after, sequence comparing software packages such as BLAST and CLUSTAL X are used for alignment of 16S rRNA gene sequence. The extent of relatedness between bacterial species can be scrutinized by the construction of phylogenetic tree or dendrogram using freely available tree-making softwares such as PAUP, PHYLIP and MEGA 4 (Fig. 3a and b). The phylogenetic tree ascertains the genus to which the strain belongs and its closest neighbours i.e. those sharing the clade or showing > 97% 16S rRNA gene sequence similarity are obtained from various culture collections to perform further genotypic, chemotaxonomic and phenotypic analysis. For example strains *Sphingomonas paucimobilis* B90A, Sp+ and UT26 were discerned as separate species viz., *Sphingobium indicum* B90A, *Sphingobium francense* Sp+ and *Sphingobium japonicum* UT26 respectively (as shown in Fig. 3b) following comparative genotypic, chemotaxonomic and phenotypic analyses with *S. cholrophenolicum* ATCC 33790^T*, S. chungbukensis* and *S. paucimobilis* ATCC 29837^T [32]. An outgroup can be selected (a taxon outside the group of interest) while constructing a dendrogram (Fig 3a and b) which serves as a root depicting the evolutionary pattern of the strains[8]. The phylogenetic tree can be constructed using several methods of which Neighbor-Joining, Maximum Parsimony and Maximum Likelihood are most commonly used. These aim to find the best explanation for the given set of data. These methods deal with the same data in different ways[33,34,35] and so, different results are generated. Therefore, it is essential to use at least two methods and to select the tree which best depicts the phylogenetic relation.

c. G+C ratio

Analysis of DNA G+C ratio or mole percent of guanosine and cytosine is one of the classical genotyping methods in the bacterial systematics. The variation in the percent GC content is not more than 3% within a well-defined species and not more than 10% within a well-defined genus and it varies from 24 to 76% in the bacterial world[36].

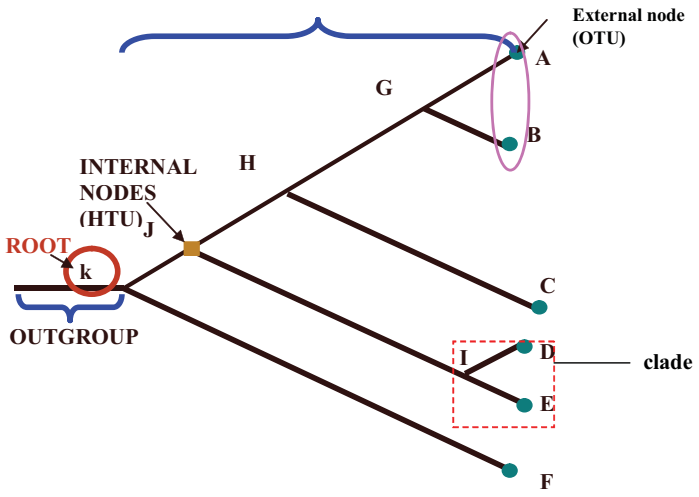
Table 1 Universal primers used for sequencing of the 16S rRNA gene*****.

PRIMERS	SEQUENCES OF THE PRIMER	BINDING POSITIONS OF THE PRIMER
	Forward Primers	
F_8	5`- AGA GTT TGA TCC TGG CTC AG -3`	8–28
F_{50}	5`- TAA CAC ATG CAA GTC GAA CG -3`	50–70
F_{341}	5`- CTA CGG GAG GCA GCA GTG GG -3`	341–361
F_{518}	5`- CAG CAG CCG CGG TAA TAC -3`	518–536
F_{908}	5`- AAA CTC AAA GGA ATT GAC GG -3`	908–928
F_{1053}	5`- AAG GAG GTG ATC CAG CCG CA -3`	1053–1073
	Reverse Primers	
R_{361}	5`- CCC ACT GCT GCC TCC CGT AG -3`	361–341
R_{536}	5`- GTA TTA CCG CGG CTG CTG -3`	536–518
R_{928}	5`- CCG TCA ATT CCT TTG AGT TT -3`	928–908
R_{1073}	5`- ACT AGC TGA CGA CAG CCA TG -3`	1073–1053
R_{1407}	5`- ACG GGC GGT GTG TAC -3`	1407–1392
R_{1542}	5`- AAG GAG GTG ATC CAG CCG CA -3`	1542–1522

*****Data from Edwards *et al.* (1986)[73].

The nucleotide postions in the 16S RRNA gene given in the table refer to the positions in *E.coli* 16S rRNA gene.

(3a)



(3b)

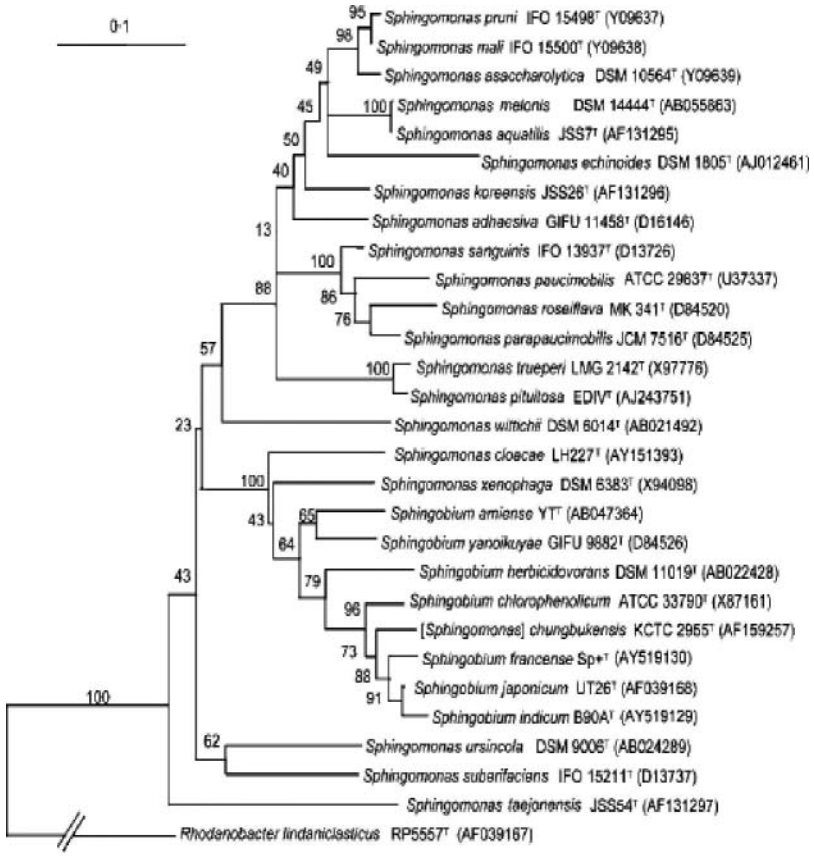


Fig. 3a A simple representation of dendrogram with 'K' species taken as an outgroup; nodes (branch points) are categorized as internal nodes or HTU (Hypothetical Taxonomic Unit which include G, H, I, J) and external nodes or OTU (Operational Taxonomic Unit which include A, B, C, D, E, F); I, D and E together represent a clade (a clade includes an ancestral species and all its descendents).

Fig 3b. Phylogenetic tree based on nearly complete 16S rDNA sequences (1444 aligned positions) showing relationship between strains; *Sphingobium indicum* B90A*, Sphingobium japonicum* UT26 *and Sphingobium francense* Sp+ The tree was constructed by neighbour-joining method and rooted using *Rhodanobacter lindaniclasticus* as an outgroup. The scale bar indicates 0.1 nucleotide substitution per nucleotide position. The GenBank accession number for the 16S rRNA gene sequence of each reference species is shown in parenthesis. (Data adapted from Pal *et al.* 2005[32]).

d. DNA-DNA reassociation

DNA-DNA hybridization or DNA-DNA reassociation technique is based on a comparison between whole genome of two bacterial species. The application of DNA-DNA reassociation method in bacterial classification for delineation of species was evaluated by a committee on systematics[37]. According to their recommendation, bacterial species generally would include the strain with 70% or greater DNA-DNA hybridization values with 5°C or less ΔTm values and both the values must be considered. However, it must be noted that this technique gives the relative % of similarity but not the actual sequence identity.

The technique is based on the fact that at high temperatures, DNA can be denatured, but the molecule can be brought back to its native state by lowering down the temperature (reassociation). It is based on three parameters i.e., i) G + C mol % , ii) the ionic strength of the solution and iii) the melting temperature of DNA hybrid (Tm). Tm is the only variable parameter out of three (as ionic strength can be kept constant). Therefore, more the similarity between the heteroduplex molecule, more temperature will be required to separate it (high Tm value). Stringency of this technique is also dependent upon the salt and formamide concentration. Many different methods for DNA-DNA hybridization have been compared by Mora in 2006[38], but the crux of the technique remains more or less same.

DNA-DNA hybridization experiment has also been criticized from time to time by several workers due to its high experimental error, inaccurate reproducibility of the result and failure to generate cumulative database[39]. There is a yearning to replace this method with other more accurate and reliable techniques but its use cannot be avoided. Till date more than 5000 bacterial species have been successfully delineated on the basis of this technique[38].

Another method that is expensive and slated to overcome the shortcomings of DNA-DNA hybridization is DNA microarray. It too involves hybridization of DNA, but instead of whole genomic DNA, fragmented DNA is used. Numerous DNA fragments can be hybridized on a single microarray and generates portable data. This method gives resolution upto strain level and has been used in detecting virulence among the strains of pathogenic bacteria by identifying the strain-specific unique regions[40].

II. Chemotaxonomy

The term chemotaxonomy refers to application of analytical methods for collecting information on different chemical constituents or chemotaxonomic markers of bacterial cells in order to group or organize them into different taxonomic ranks[3,4]. The principle of chemotaxonomy is based on uneven distribution of these markers among different microbial groups[41]. The use of these analytical methods varies from group to group. The most commonly used chemical markers include cell wall/membrane components such as peptidoglycan[42], teichoic acids, polar lipids, composition and relative ratios of fatty acids, lipopolysaccharide, isoprenoid quinones and polyamines[43,44]. Teichoic acids are the polymers of glycerol and are specifically used for characterizing Gram-positive bacteria. Respiratory quinones which belong to a class of terpenoid lipids are constituents of bacterial plasma membrane and are valuable in microbial systematics[45]. For instance, all sulphur-containing bacteria are characterized by the presence of caldariellaquinone, an unusual terpenoid[46]. Analysis of these compounds by using different chromatographic techniques can successfully delineate the bacteria upto the rank of genus.

III. Phenotypic Methods

Phenotype is the observable expression of genotype and it includes morphological, physiological and biochemical properties of the organism. Before the advancement in molecular techniques, bacterial taxonomy was solely based on comparative studies of the phenotypic features and this practice was directly linked to laboratory pure cultures. Therefore, it was biased towards aerobic heterotrophic microorganisms[3,4]. Traditional phenotypic tests used in classical microbiological laboratories for this kind of analysis include characteristics of the organism on different growth substrates, growth range of microorganisms on different conditions of salt, pH and temperature and susceptibility towards different kind of antimicrobial agents etc.

One of the major disadvantages with phenotypic methods is the conditional nature of gene expression wherein the same organism might show different phenotypic characters in different environmental conditions. One must note that phenotypic data must be compared with similar set of data from type strain of closely related organism(s). Reproducibility of results between different laboratories is another problem, therefore, only standardized procedure should be used during execution of experiment[47,48].

The classical approach of phenotypic evaluation using Numerical taxonomy involves conversion of characteristics of taxonomic entities into a digital form coupled with computer assisted grouping of the organisms after providing equal weight to all the characters. In short, it is known as phenetic evaluation of phenotypic data. This involves selection of strains which represent fresh isolates of a type strain from the culture collection so that little modification has occurred under laboratory conditions. After selection of

appropriate strain for comparison, a set of routine tests must be performed. Standardization of treatment and conditions of inoculation and incubation is essential before performing the tests[49]. Data obtained from above analysis is generally represented as +ve and –ve or in binary format (0 and 1). Finally cluster analysis should be performed using different measure of similarity and distance and different clustering algorithms. Numerical taxonomy has supported the development of a stable classification system for prokaryotes and the database generated in this way is used for information storage and identification system[50].

Some of the standard examples that follow the scheme to delineate species (as discussed above) can be read in Bala *et al.* 2004[51], Pal *et al*. 2005[32], Majumdar *et al*. 2006[52], Prakash and Lal 2006[53], Prakash *et al.* 2007[54].

Pitfalls of polyphasic approach

Though a number of published bacterial species have been described by using the polyphasic approach, its use as a standard reference has been a matter of debate of recently. Criticisms to this approach started appearing in 1990's[55]. There have been reports wherein 16S rRNA identity is not found to confirm with the DNA-DNA hybridization studies[14,56] and generate data which is misleading!

Whole Genome Sequencing and Bacterial Systematics

It was generally believed that the chances of an organism being able to use rRNA from any other organism are very low but there are evidences that functional ribosomes can be reconstituted from different organisms[57-60]. Each ribosomal operon of an organism can be replaced with those of another species[61,62] and divergent rRNA operons can coexist in the same genome[63,64]. Not only this, divergent operons showing extensive recombinations have been observed that are functional[62,64,65]. Thus, the present concept of hierarchical classification based on single gene or a cluster of genes at times might not reflect the accurate scenario of evolution. Even conserved genes are subjected to deletions, duplications, mutations, recombinations and lateral gene transfer. The genome complexity of an organism is actually the complete history of genetic recombinations and other nucleotide sequence drifts that occurred during the course of evolution. Further, it is known that genetic diversity among strains of the same species is far greater than was expected earlier. Even the genome size of 20 *Escherichia coli* strains varies from 4.6 to 5.5 Mb which means a million base pairs are present in some *E. coli* strains which are missing in others[66]. The genome sequence analysis of *E. coli* O157:H7 by Perna and colleagues, in 2001 revealed that the strain possesses 1300 strain specific genes compared to *E. coli* K-12 thus indicating that two members of the same species can show 30% variation in their gene content[67]! Such examples support the fact that microbial genome is a dynamic unit shaped by multiple parameters. Such high extent of disparity among the strains of same species has even contradicted the notion of DNA-DNA hybridization and hence, alarms a need for complete genome sequencing for further clarification of phylogenetic relationship between organisms.

In the present genomic era, the availability of complete genome sequence of more then 1000 bacteria provides an opportunity to reanalyze phylogenetic evolution. In order to represent a consolidated picture of evolution based upon genome sequence data 'supertree approach' was followed. Supertree approach converges the information and significant interpretations of hundred of trees from orthologous gene families in one supertree. The concept of supertree approach has opened a new direction of bacterial phylogeny termed as phylogenomics. Phylogenomic analysis of genome sequence data of 45 organisms using supertree approach based on core genes inferred several differences with rDNA phylogeny, specially on the evolutionary position of hyperthermophilic bacteria[68] and further attempts have been made with a shift from gene tree to organismal phylogeny in case of γ proteobacteria[69].

Perhaps, the 16S rRNA gene data coupled with whole genome sequence analysis will provide an enlightened picture of bacterial evolution keeping controversies at lower end. The role of horizontal gene transfer in microbial evolution as evident from the mosaic nature of the genomes has forced the scientific community to re-assess the present system of prokaryotic evolution and taxonomy[70]. The exact quantification of the horizontal gene transfer is still a matter of debate but the analysis based on nucleotide or codon composition revealed that 17% of the gene content of bacterial genome is alien in origin with a minor contribution from mobile elements[71]. Keeping in view the significance of horizontal gene transfer in determining genome of microbes a new line of comprehensive evolution coupling vertical and horizontal gene transfer has been drawn for further authentic study of bacterial phylogeny. The first attempt of reconstructing evolutionary network considering vertical and horizontal gene transfer termed as 'net of life' has been performed by Kunin *et al.* 2005[72]. Further developments will surely make significant clarity in the science of bacterial evolution covering the broad range concept of polyphasic approach.

A Road Map for Performing Taxonomical Inference

An easy way to draw taxonomical inference from newly isolated strain(s) of a niche is depicted in the Fig. 2. At first,

all the isolates of a nice should be screened using one of the methods like Pulse Field Gel Electrophoresis (PFGE), Restriction Fragment Length Polymorphism (RFLP), Rep-PCR etc. (described in the DNA based typing method section) in order to avoid the strain duplication and thus to save time, cost and efforts. After that, a phylogenetic tree should be constructed using at least two different methods with full length 16S rRNA gene sequences. Strain(s) of interest must be delineated with all other members of the group showing ≥97% 16S rRNA gene sequence similarity on the basis of DNA-DNA hybridization and comparative phenotypic data. Inferences drawn from above methods be confirmed by chemotaxonomy (analysis of chemical markers) and also by other genetic means like accessing G+C content or total genome sequences. Finally if strain under consideration qualifies all these conditions is it a new species, it is named as per the international norms set for this purpose. It is deposited in two international culture collections and generally published in reputed journals like International Journal of Systematic and Evolutionary Microbiology (IJSEM). Fig. 1 and Fig. 2 depict the steps that are to be followed to describe a new bacterial species.

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