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NEW STRATEGIES ON MOLECULAR BIOLOGY APPLIED TO MICROBIAL SYSTEMATICS

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SUMMARY

Systematics is the study of diversity of the organisms and their relationships comprising classification, nomenclature and identification. The term classification or taxonomy means the arrangement of the organisms in groups (rate) and the nomenclature is the attribution of correct international scientific names to organisms and identification is the inclusion of unknown strains in groups derived from classification. Therefore, classification for a stable nomenclature and a perfect identification are required previously. The beginning of the new bacterial systematics era can be remembered by the introduction and application of new taxonomic concepts and techniques, from the 50's and 60's. Important progress were achieved using numerical taxonomy and molecular taxonomy. Molecular taxonomy, brought into effect after the emergence of the Molecular Biology resources, provided knowledge that comprises systematics of bacteria, in which occurs great evolutionary interest, or where is observed the necessity of eliminating any environmental interference. When you study the composition and disposition of nucleotides in certain portions of the genetic material, you study searching their genome, much less susceptible to environmental alterations than proteins, codified based on it. In the molecular taxonomy, you can research both DNA and RNA, and the main techniques that have been used in the systematics comprise the build of restriction maps, DNA-DNA hybridization, DNA-RNA hybridization, sequencing of DNA sequencing of sub-units 16S and 23S of rRNA, RAPD, RFLP, PFGE etc. Techniques such as base sequencing, though they are extremely sensible and greatly precise, are relatively onerous and impracticable to the great majority of the bacterial taxonomy laboratories. Several specialized techniques have been applied to taxonomic studies of microorganisms. In the last years, these have included preliminary electrophoretic analysis of soluble proteins and isoenzymes, and subsequently determination of deoxyribonucleic acid base composition and assessment of base sequence homology by means of DNA-RNA hybrid experiments beside others. These various techniques, as expected, have generally indicated a lack of taxonomic information in microbial systematics. There are numberless techniques and methodologies that make bacteria identification and classification study possible, part of them described here, allowing establish different degrees of subspecific and interspecific similarity through phenetic-genetic polymorphism analysis. However, was pointed out the necessity of using more than one technique for better establish similarity degrees within microorganisms. Obtaining data resulting from application of a sole technique isolatedly may not provide significant information from Bacterial Systematics viewpoint.

KEYWORDS: Microbial systematics; New strategies; Molecular biology.

INTRODUCTION

Systematics is the study of diversity of the organisms and their relationships comprising classification, nomenclature and identification[41]. The term classification or taxonomy means the arrangement of the organisms in groups (rate). Nomenclature is the attribution of correct international scientific names to organisms. Identification is the inclusion of unknown strains in groups derived from classification. Therefore, classification for a stable nomenclature and a perfect identification is required previously.

The majority of modern bacterial taxonomies are based on global similarity. These taxonomies are, at times, called phenetic classifications, once they derive from similarities and differences of the phenotypic characters. Contrasting to phylogenetics, the word phenetic has no evolutionary implication, except in the context of showing the end product of evolution[73]. Phylogenetic classifications are expressions of the evolutionary relationships among organisms. They convey the degree of changing in the evolutionary lines.

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The beginning of the new bacterial systematics era can be remembered by the introduction and application of new taxonomic concepts and techniques, from the 50's and 60's. Important progress was achieved using numerical taxonomy and molecular taxonomy.

The conventional numerical taxonomy is an efficient means used to establish phylogenetic relationships in lower levels to genre[50]. Principle of numerical classification involves the generation of a great deal of data that are grouped in clusters (taxospecies) based on similarity index[72]. Firstly, equal values are attributed to all characters and, after successive confirmatory repetitions, a similarity matrix is generated or a phenogram that will allow the numerical identification of the isolated[84]. Among the samples being studied standard strains are included and their origin and classification are known what will allow the numerical comparison.

Numerical classification is based on phenetic data that are analyzed two by two. The main methods include serology contrasted with antibodies, total proteins and isoenzymes. As one could notice, these methods searched only the presence or absence of proteins in the samples confirming that numerical taxonomy evaluates phenetic data.

Molecular taxonomy, brought into effect after the emergence of the Molecular Biology resources, provided knowledge that comprises systematics of bacteria[65, 66], in which occurs great evolutionary interest, or where is observed the necessity of eliminating any environmental interference. When you study the composition and disposition of nucleotides in certain portions of the genetic material, you study searching their genome, much less susceptible to environmental alterations than proteins, codified based on it. In the molecular taxonomy, you can research both DNA and RNA, and the main techniques that have been used in the systematics comprise the build of restriction maps, DNA-DNA hybridization, DNA-RNA hybridization, sequencing of DNA, sequencing of sub-units 16S and 23S of rRNA, RAPD, RFLP, PFGE, etc. Techniques such as base sequencing, though they are extremely sensible and greatly precise, are relatively onerous and impracticable to the great majority of the bacterial taxonomy laboratories.

Several specialized techniques have been applied to taxonomic studies of microorganisms. In the last years, these have included preliminary electrophoretic analysis of soluble proteins and isoenzymes, and subsequently determination of deoxyribonucleic acid base composition and assessment of base sequence homology by means of DNA-RNA hybrid experiments near of others.

In this review we will show some aspects and applications of the following Molecular Biology techniques: One-Dimensional SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Isoenzymes, Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphism of DNA (RAPD), Ribossomic RNA Polymorphism, and Pulsed Field Gel Electrophoresis (PFGE).

ONE-DIMENSIONAL SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has emerged as one of the most efficient versatile and inexpensive techniques for the separation and resolution of bacterial proteins[22] that may be used for the establishment of the phenotypic variability and relationship within species[16, 26, 69]. The usage of SDS promotes modification of total electric charge of proteins annulling the electric positive charges and favoring differential migration because of molecular mass differences[54]. Once electrophoresis finished, that is conducted in discontinuous buffer system[48], gels may be dyed using protein dyes[22] or silver[87].

Dispositions of protein bands, along the gel, are converted into numerical values based on their relative mobilities (Rf values), that are compared 2 by 2, concerning presence or absence of such bands. These values generate a binary data matrix that, by specialized computer programs[46], provide similarity matrix that may be converted to similarity phenograms[40, 72].

In the last decades, many researches have applied this technique in the identification of clinically significant strains at the species level and its use has found a higher application nowadays. Protein patterns offer considerable potential for typing strains of clinical interest and for taxonomic purposes, especially for the studies of the qualification of biodiversity among microorganisms[8, 10, 11, 57]. Several entities could be better classified using this technique. COSTAS et al.[27] analyzed 31 isolated of *Providencia rettgeri* of different origins (feces, urine, spittle, insect) and classified them in 13 clusters, with a minimum of 84% of similarity. COSTAS et al.[28] assessed levels of similarity among 20 samples of *Enterobacter cloacae* obtained from 2 British hospitals and classified in 11 clusters with a minimum of 90% of similarity. TANNER et al.[78] issued an article in which identified, through SDS-PAGE and DNA-DNA hybridization, 12 strains of *Bacterioides* isolated from advanced periodontal lesions (with 90% of similarity, among them) and due to the low similarity to other species, *Bacteriodes forsytus* was suggested to this new specie.

This technique is still employed because of its low costs, simplicity and possibility of reducing the number of isolates that would be analyzed by other molecular methods more complex (VANCANNEYT et al.[81], 1991).

ISOENZYMES

In Bacteriology, the usage of isoenzymes in organisms classification refer to articles issued in the 60's and has assisted Systematics and Taxonomy of several genera and species[6]. Strains of *Escherichia coli* were intensively assessed concerning genetic diversity, in isolated of urinary infection[20] and other sources[18, 19]. *Haemophilus influenzae*[55], *Neisseria*[17], *Pseudomonas* of medical interest[49] and of agronomic interest[7], *Rhizobium*[88], among others.

FOTTREL[32] reports that Market & Møller in 1959 were the first ones who observed the occurrence of multiple forms of an enzyme converting the same substratum to which the denomination of isozyme (or isoenzyme). HARRIS[42] explains isoenzymes as being variations of the structure of an enzyme, genetically determined, occurring within the same specie. It may be due to the fact that more than one genic locus codifies separate versions of one enzyme or due to the fact of the existence of multiple alleles occurring in the same locus (allelic polymorphism). PRAKASH et al.[60] designated allelic variants of codified enzymes in the same genic locus as allozymes (or alloenzymes) attributing the isoenzyme designation to those that are codified in different loci. Biochemistry Nomenclature Commission of IUPAC-IUB suggested a classification to multiple enzymatic forms in 1977, divided in seven groups: 1) genetically independent proteins; 2) polypeptide chains, not linked in a covalent way; 3) allelic genetic variants; 4) proteins conjugated to other groups; 5) proteins derived from a polypeptide chain; 6) polymers of one sole sub-unit, and 7) different structure forms. Analyzing this classification, DIXON & WEBB[30] conclude that groups 1, 2 and 3 are true isoenzymes, while groups 4, 5 and 6 are secondary isoenzymes derived from posttranscriptional modifications of the products of the same gene. Genetically independent proteins (group 1) are products of separated genes, but in some cases these genes may be originated from the duplication of a common ancestor, followed by independent variants. The authors above quote publications of Holmes & Scopes in 1974, Masters & Holmes in 1975 and the review of Whitt, Shaklee & Markert in 1975, developed from variations of lactate dehydrogenase enzyme (E.C. 1.1.1.27) in different animal species, became confirmatory of this theory. Isoenzymes may be evidenced after extraction and not denaturant electrophoresis of the bacterial protean content, in gels of starch or polyacrylamide (ALFENAS et al.[4]) that are "developed" using solutions of substratum and color indicatives (SELANDER et al.[68]).

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

The technique of Restriction Fragment Length Polymorphism (RFLP) is a variation of the technique of DNA-DNA hybridization that is based in the capacity of two complementary DNA strands recognize and recombine by means homology on their sequences of nucleotides, with great applicability in the bacterial systematics. The polymorphism observed in the RFLP technique occurs because the DNA of genetically separate individuals differ in the sequence of nucleotides along the DNA[31]. The presence or absence of specific sequences from 4 to 8 pairs of bases, recognized and cleaved by restriction enzymes, may vary among different individuals, generating polymorphism. Differences in the individuals DNA sequence may also result from insertions, deletions or from other rearrangements (translocations, inversions) that alter the distance among pairs of restriction sites. Undergoing a cleavage by a restriction enzyme, the DNA of genetically separate individuals is cut in the restriction sites generating fragments of different sizes. This way, the genetic base of the observed polymorphism results from mutations in the restriction sites or from deletions, insertions and other rearrangements among these sites.

The basic principle of this technique consists of enzymatic digestion of the DNA, by endonucleases, followed by electrophoresis in gel of agarose that will be subjected to a transference to nylon membrane (Southern Blot). As restriction enzymes cut the DNA in many fragments (thousands), if we process a development of the membrane using an ordinary indicator, as ethidium bromide or the like, we observe a electrophoretic track proceeding from enzymatic digestion in several points of the length of genome. Portions of the DNA radioactively marked or chemiluminescent agent (probes), may hybridize themselves with homologous segments of the immobilized DNA in the membrane. Once taken out the excess of probe that didn't go through hybridization, the exposition of the membrane to a radiographic or photographic film, it will sensitize the radiographic film in portions where the probe settles appearing bands that may represent genetic polymorphism. RFLP technique allows obtaining a great quantity of electromorphic profiles varying restriction enzymes and probes. RFPL markers show genetic variation in the nucleotide sequence of the region that codify genic products and, on a smaller scale, in non-transcriptable regions, while isoenzymes are restrict to transcriptable regions.

Unlike isoenzymes, the number of RFLP markers is practically unlimited and allelic polymorphism is much higher in each locus[33]. COOK et al.[24] used RFLP technique in order to analyze phylogenetic relationships among 62 strains of *Pseudomonas solanacearum*, phytopathogenic bacteria. Genomic DNA was digested by restriction enzymes *Eco*R I and *Bam*H I and the fragments were hybridized by Southern Blot with DNA probes. The bands obtained were analyzed and allowed the calculation of similarity coefficient among different strains. Two separate groups were identified: the first one includes race 01 (biotypes 3, 4 and 5) and the second one includes race 01 (biotype 01) and races 02 and 03. The average similarity coefficients were 78% and 62% respectively in each group and among the groups 13.5%. ALMEIDA et al.[5] used sequences of a bacteriophage as probe in order to characterize *Vibrio cholerae* after digestion with *Hind* III, obtaining ten RFLP patterns among 58 isolated. This kind of study can also be carried on with a specific segment of DNA, for instance, the DNA of the 16S rRNA, previously enlarged through PCR technique. NORMAND et al.[56] used enlargement of codifier genes segment of 16S rRNA of species of *Frankia* sp, an actinomycete fixer of nitrogen, in order to establish phylogenetic relationships among strains of these organisms. The phylogenetic tree was established which show the great existing diversity among physiological races within the genre and alterations were proposed within the *Frankiacea* family.

The ability in to associate with other techniques, as amplification by PCR or use of rRNA probes, seems to be the main advantage of the RFLP allowing the increasing of

sensibility and applicability in researches of microbial variability.

RANDOM AMPLIFIED POLYMORPHISM OF DNA (RAPD)

Random Amplified Polymorphism of DNA (RAPD) technique is an evolution of using markers based on Polymerase Chain Reaction (PCR). PCR is a powerful technique that involves enzymatic synthesis *in vitro* (conduced in apparatus called "thermal cyclers") of millions of copies of a specific segment of DNA in the presence of DNA polymerase enzyme. PCR reaction is based on enzymatic amplification after annealing of "primers" (starters, from the edges 5) that limited the sequence of the double strand which is the aim of enlargement[63]. These primers are artificially synthesized in a way that the nucleotide sequences are complementary to specific sequences which go along the aim region. In the RAPD technique only one "primer" is used, arbitrary, while in the PCR classic reaction two primers are used with known aim sequences[81]. In order to occur enlargement of a RAPD fragment in the genome in question, two sequences of complementary DNA to the arbitrary primer must be near enough (more than 4,000 base pairs) and in opposite orientation, in a way to allow exponential enlargement of the aim segment through Taq polymerase. Once finished the enlargement (after several cycles), electrophoresis is processed in agarose gel with later development through ethidium bromide, in ultraviolet light or polyacrylamide gel of high resolution, and visualization through radioautograph[13]. The appearing of electrophoretic bands allow the assessment of the molecular nature of the polymorphism to RAPD loci. Experimental evidences lead to the fact that differences of only one pair of bases (point mutations) is enough to cause primer non-completeness with the linking site and, this, way, impede the enlargement of a segment[83]. Other sources of polymorphism may include deletions of starting sites or insertions that place two starting sites in an adjacent position, in a greater distance which Taq polymerase is able to running. This way, the genetic polymorphism detected by RAPD markers has a binary nature, that is, the enlarged segment (expressed by band in the gel) may be present or absent.

Markers based on RAPD fingerprints has permitted the identification or classification of numberless bacterial entities in several fields: in agriculture, BROUSSAU et al.[12] could identify several strains of *Bacillus thuringiensis*, an entomopathogenic bacteria used as biological insecticide. In odontology, MENARD et al.[53] established patterns to the subspecific identification and classifiction of *Porphyromas gengivalis*, anaerobic bacteria implied in the development of the periodontal infections. In medicine, several genera were assessed. *Helycobacter pylori*, main organism involved with appearing and development of gastric ulcers, was studied what concerns its diversity in samples of clinical material by AKOPYANZ et al.[1]. *Campylobacter* isolates from diverse anatomic sites were analyzed by MAZURIER et al.[52], using such technique. In this same year, MAZURIER et al.[51] issued an article in which they compared RAPD profiles to other methods of lineage classification for isolates of *Listeria* from clinical material.

Nowadays, the development of more efficient thermal cyclers and better reagents and PCR primers, have increased the quality of the RAPD analyses, and this technique has shown be a powerful tool for applications in taxonomic studies and epidemiological investigations.

RIBOSSOMIC RNA (rRNA) POLYMORPHISM

Special attention has been given to ribotyping. This methodology is based on the idea that the ribossomic RNA (rRNA) alters very slowly as time goes by and this property makes it an extraordinary "biological clock" or "molecular chronometer" through which different genera present different DNA sequences, they may be compared in phylogenetic studies[44, 84]. In the *Escherichia coli* DNA, there are seven separate "operons" that collaborate in the rRNA synthesis that represents 70% to 80% of the total RNA of this species. This nucleic acid is composed of three sub-units: 16S, 23S and 5S. Sub-unit 16S is synthesized from a sequence of nearly 1,500 nucleotides. Sub-unit 23S is synthesized from a sequence of nearly 3,000 nucleotides. Sub-unit 5S is synthesized from a sequence of nearly 120 nucleotides. Among these three segments there are intergenic spacer regions. These sub-units bigger and smaller are conserved regions, at subspecific level and highly variable, at subgeneric level. rRNA genes were largely used in order to detect polymorphism among bacteria given the fact that they represent about 0.1% of the genome and are highly conserved[75]. This property was first observed by GRIMONT & GRIMONT[37] to identify sub-group within a species, but the word "ribotyping" was used by STULL et al.[75] in order to describe a method of typing by RFLP in studies of molecular epidemiology for a wide range of bacterial species.

Probes may be rRNA molecules prepared from a representative strain of the study group or, in some cases in which there is enough similarity of sequences, rRNA obtained commercially from *E. coli*, may be used as probe[3, 39].

Concerning ribotyping technique, DNA is extracted from bacterial cells and digested by restriction endonucleases. Fragments are separated by electrophoresis in agarose gel and transferred to the nylon membrane or cellulose acetate ("Southern Blot" technique). These fragments are complementary to the rRNA probe that are observed then, after hybridization, by radioactivity or chemiluminescence[38, 39].

Usage of ribotyping has been helping genotypic characterization of streptococci of medical and odontological importance[2, 62]. TEE et al.[79] have observed the occurrence of 77 different patterns within 126 clinical isolated of *Helicobacter pylori*. In a study searching for evaluating differences at subspecific level to clinical isolated of *Staphylococus epidermitis*, IZARD et al.[43] obtained 11 ribotypes from 86 isolated. SMITH & CALLIHAN[70], in a study about subspecific variability of the *Bacterioides* genus, used ribotyping to compare 7 species of medical and veterinary.

Ribossomic RNA (rRNA) Polymorphism method has been used mainly in phylogenetic studies, when the researchers want

to establish the relationship among some groups of microorganisms. With the emergence of techniques more sensible to genetic variation, as RAPD technology, the studies involving epidemiological interest have not more employed the use of ribossomic RNA as genetic marker.

PULSED FIELD GEL ELECTROPHORESIS (PFGE)

With similar relevance, the Pulsed Field Gel Electrophoresis (PFGE) has been providing a very important taxonomic knowledge. This technique relies on the concept that the limit of the size of DNA fragments separable by conventional electrophoresis in agarose (about 50 kb) may be increased by introducing pulses or changes in the direction of the electric field[45]. PIZZIRANI-KLEINER & AZEVEDO[59] mention that over 50-60 kb DNA molecules have electrophoretic mobility that are independent of their respective sizes, that is, they all migrate together in the gel, once they present greater sizes than the gel pore diameters so that they dispose themselves longitudinally to the pores.

It was SCHWARTZ & CANTOR[67] who solved the matter of electrophoretic separation of big DNA segments. These researchers observed that alternating the direction of the electric field, they could cause the molecules to migrate differentially. Applying a variable electric field (here derives the word 'pulsed'), the DNA molecules move diagonally, in "zigzag", this way, being able of isolating themselves one from another because of their molecular mass. Several electrode configurations were tested aiming improvement of the technique: OFAGE – Orthogonal Field Alternation Gel Electrophoresis[14]; FIGE – Field Inversion Gel Electrophoresis[15]; CHEF – Contour-Clamped Homogeneous Electric Field[23], as well as variations of each of these methods (i.e. usage of vertical gels). According to BIRREN & LAI[9] there are other configurations: TAFE (Transverse Alternating Field Electrophoresis), RGE (Rotating Gel Electrophoresis), PACE (Programmable Autonomously Controlled Eletrodes), ZIFE (Zero Integrated Field Electrophoresis) and ST/RIDE (Simultaneously Tangential/ Rectangular Inversion Decussate Electrophoresis).

That technique allows obtaining electrophoretic profiles of high molecular mass DNA, for instance chromosomes, allowing the elaboration of eucariotic karyotype whose chromosomes are very small (fungus, yeasts, protozoon etc.). Still, it allows long fragment separation from bacterial chromosome recently digested by restriction endonucleases of rare cleavage sites[44]. Using this technique, it's feasible to make physical maps to several bacterial genera, once endonucleases which provides great restriction fragments are used[47]. These physic maps may provide important information as size of the genome[71, 76], chromosome conformation[29, 58, 77] or genetic maps when hybridization is done with "cloned" probes from restriction fragments[41, 80].

Electrophoresis in pulsed field has been used in epidemiological research of strains of *Escherichia coli* apart from health professionals[25]. GORDILLO et al.[36] could assess the variability of isolated of *Escherichia coli* of an epidemic outbreak comparing to an invasive lineage (EIEC), after a previous digestion by *Xba* I. CHANG & TAYLOR[21] have made restriction maps and determined genome size of *Campylobacter hyointestinalis* in clinical isolated. Both publications used *Scal* I endonuclease, showing the flexibility of usage of this technique. GOERING & DUENSING[34] used PFGE associated with hybridization through probes to rRNA genes in order to classify isolates of *Staphylococcus* of epidemiological interest. PREVOST et al.[61], working with *Staphylococcus aureus* methicilin-resistent, obtained 26 separate electrophoretic patterns in 239 isolated, after digestion with *Sma* I. Still, these authors have preconized the superiority of this PFGE technique to the characterization of these organisms when compared to the ribotyping technique.

There are numberless techniques and methodologies that make bacteria identification and classification study possible, part of them described here, allowing establish different degrees of subspecific and interspecific similarity through phenetic-genetic polymorphism analysis. GOMES[35], however, points out the necessity of using more than one technique for better establish similarity degrees within microorganisms. Obtaining data resulting from application of a unique technique separately may not provide significant information from Bacterial Systematics viewpoint.

RESUMO

Novas estratégias em biologia molecular aplicadas à sistemática microbiana

Sistemática é o estudo da diversidade dos organismos e suas relações, compreendendo classificação, nomenclatura e identificação. O termo classificação ou taxonomia relaciona-se ao arranjo dos organismos em grupos, nomenclatura é a atribuição de nomes científicos internacionais corretos aos organismos e identificação é a inclusão de linhagens desconhecidas em grupos derivados da classificação. Portanto, a classificação requer previamente, uma nomenclatura estável e uma perfeita identificação. O limiar da nova era da Sistemática bacteriana, pode ser atribuído à introdução e aplicação dos novos conceitos taxonômicos, a partir das décadas de 1950 e 1960. Progressos importantes foram conseguidos empregando-se recursos de taxonomia numérica e a taxonomia molecular. A taxonomia molecular surgiu com a emergência dos recursos da Biologia molecular, provendo conhecimentos que abrangem a Sistemática de bactérias, na qual existe grande interesse evolutivo, ou onde é observada a necessidade de eliminação de quaisquer interferências ambientais. Quando estudamos a composição e disposição de nucleotídeos em determinadas posições do material genético, estamos procurando conhecer seu genoma, muito menos susceptível às alterações ambientais que as proteínas codificadas por ele. Na taxonomia molecular, pode-se pesquisar tanto o DNA quanto o RNA, e as principais técnicas que tem sido empregadas na Sistemática compreendem a construção de mapas de restrição, hibridação DNA-DNA e DNA-RNA, seqüenciamento do DNA, seqüenciamento das

subunidades 16S e 23S do rRNA, RAPD, RFLP, PFGE, etc. Técnicas baseadas em seqüenciamento, embora extremamente sensíveis e de grande precisão, são relativamente caras e impraticáveis para a grande maioria dos laboratórios de taxonomia bacteriana. Algumas outras técnicas tem sido aplicadas ao estudo de microrganismos, nos últimos anos elas incluíram preliminarmente, a análise eletroforética de proteínas solúveis e isoenzimas e subseqüentemente a determinação da composição das bases do DNA e a determinação da homologia das seqüências de bases através de experimentos com hibridação DNA-RNA, entre outros. Essas várias técnicas, tem mostrado a carência de informação taxonômica na Sistemática bacteriana. Existem inúmeras técnicas e metodologias que tornam possíveis a identificação e classificação bacteriana – parte delas descritas nessa revisão – permitindo o estabelecimento dos diferentes graus de similaridade em níveis subespecífico e interespecífico, através da análise do polimorfismo fenético. Contudo foi apontada a necessidade do emprego de mais de uma técnica para melhor se estabelecer graus de similaridade entre microrganismos. Dados obtidos à partir de uma única técnica isolada, podem não prover informação suficiente para a Sistemática bacteriana.

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