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How are gene sequence analyses modifying bacterial taxonomy? The case of *Klebsiella*

Summary. Bacterial names are continually being changed in order to more adequately describe natural groups (the units of microbial diversity) and their relationships. The problems in *Klebsiella* taxonomy are illustrative and common to other bacterial genera. Like other bacteria, *Klebsiella* spp. were isolated long ago, when methods to identify and classify bacteria were limited. However, recently developed molecular approaches have led to taxonomical revisions in several cases or to sound proposals of novel species. [**Int Microbiol** 2004; 7(4):261-268]

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Introduction

Historically, the classification of *Klebsiella* species, like that of many other bacteria, was based on their pathogenic features or origin. Later, taxonomic keys were proposed that included characteristics such as substrate utilization and enzymatic activities. A great confusion has prevailed in *Klebsiella* nomenclature [27], with new bacteria being synonymous with species already proposed [19,23,44] or with the reclassification of some species even within other genera [14]. The bacterial genus *Klebsiella* was designated in honor of the German microbiologist Edwin Klebs by Trevisan in 1885, who also described the *Klebsiella pneumoniae* species in 1887. *Klebsiella* is a well-known enterobacterial genus and *K. pneumoniae* has become a model organism in microbiology, mainly for the study of nitrogen fixation [28,47], in which ammonium is produced from gaseous dinitrogen. *Klebsiella variicola* may also serve as a model to define a bacterial species (see below). Although *Klebsiella* species are widely distributed in water, soil, and plants as well as in sewage water, it is the human pathogens that have rendered the genus notorious, and it is thought that the colonization of humans occurs from diverse sources [38].

Pathogenic *Klebsiella* strains

Bacteria belonging to the genus *Klebsiella* are frequently associated with urinary tract infections and intra-hospital septicemia and pneumonia. They are important hospital-acquired pathogens with the potential of causing severe morbidity and mortality in hospitalized patients. Several outbreaks of infections have been caused by *K. penumoniae*

multidrug-resistant strains. It is estimated that *Klebsiella* now accounts for 8% of nosocomial infections in the United States and Europe and the frequency is increasing [44]. *Klebsiella* strains colonize the mucous membranes of mammals. In humans, they are found in the epithelia of the nose and pharynx as well as in the intestinal tract. *Klebsiella* has also been associated with arthritis in humans, with mastitis in bovines [19], with atrophic chronic rhinitis, and even with hemorrhagic diarrheas and fatal infections. Immunization of people at high-risk of *Klebsiella* infection has been proposed [44].

Klebsiella in plants

Klebsiella species are found naturally associated with plants but do not cause disease. Strains of *K. planticola* and *K. pneumoniae* have been isolated from rice [30] and other plants [1,21]. The association of nitrogen-fixing bacteria with plants is ubiquitous in nature. The practical reason for studying these bacteria is that they provide a portion of the nitrogen needed by the plants, diminishing the need for the use of chemical fertilizers in agricultural fields [26,55]. It has been demonstrated that strains of *K. pneumoniae* penetrate wheat and maize [8] and, once inside the roots, produce nitrogenase, the enzyme involved in nitrogen fixation [9], although in maize an additional carbon source is required [9]. In contrast, in wheat, plant N-deficiency is relieved by *Klebsiella* providing N, which enters the plant and incorporates into chlorophyll [24]. Experiments in which maize was inoculated with *K. pneumoniae* have been carried out in agricultural fields in the United States (Triplett, personal communication). It has been shown that pathogenic *Klebsiella* strains can colonize the surfaces of potato and lettuce [29], and it has been suggested that plants could act as reservoirs of *Klebsiella* opportunistically pathogenic to humans. Whereas most clinical isolates belong to *K. pneumoniae*, *K. oxytoca* [44] and *K. granulomatis* (the supposed agent of donovanosis, a genital ulcer) [7], some correspond to *K. planticola* and *K. terrigena*. Virulence factors were found less frequently in isolates of *K. planticola* [43], and they were not present in *Klebsiella* from maize [13].

DNA-DNA hybridization

DNA-DNA hybridization studies provide valuable information for bacterial classification [54]. However, even these analyses have their limitations for use in defining species (see below). By total DNA-DNA hybridization [17,25], at least five groups were defined for *Klebsiella*: *K. pneumoniae*

[with three subspecies, subsp. *pneumoniae*, subsp. *ozaenae* (after ozaena, which is a chronic atrophic rhinitis) and subsp. *rhinoscleromatis* (after rhinoscleroma, a granulomatose chronic disease); *K. oxytoca*, *K. planticola*, *K. terrigena*, and *K. mobilis* (synonymous with *Enterobacter aerogenes*]. It is still not settled whether *K. ornithinolytica* is an independent group, but the evidence points to it belonging to *K. planticola* [5]. Since *K. granulomatis* has not yet been cultured, there are no data on total DNA-DNA hybridization to the other species [7].

DNA-DNA hybridization has the disadvantage that it does not distinguish the differences due to genome size or plasmid content. Results may vary largely depending on the hybridization conditions and the methodology used [62]. In addition, it is limited by the availability of strains to be compared, whereas sequence databases are enlarging and cover a wide spectrum of bacteria, even non-cultured ones.

16S ribosomal RNA gene analysis

Ribosomal RNA gene sequences have been extremely useful in defining bacterial relationships (including those of *Klebsiella* [3]) [53] and especially in identifying environmental or non-cultured isolates [46]; however, their value for delineating closely related species seems limited [57,59]. Furthermore, trees based on 16S ribosomal RNA genes are ambiguous in Enterobacteria and differ from one publication to another in the relative position of several genera [reviewed in 23,18], perhaps because 16S rRNA genes seem to be subject to processes of recombination and gene conversion [20,37] and different sequences of these genes may sometimes be found within a single species [56]. In *Escherichia coli*, *Salmonella* [10,20] and *Klebsiella* [50], there are seven copies of ribosomal genes.

Can other gene markers be of use instead?

To clarify the genetic relationships within Enterobacteria, sequences of *rpoB* (which codes for the β-subunit of RNA polymerase) have been used. It has been discussed that the genus *Klebsiella* is polyphyletic [12,23,40], and recently *K. planticola*, *K. terrigena* and *K. ornithinolytica* have been transferred to the new genus *Raoultella* based on *rpoB* phylogenies [14]. It is surprising that this reclassification was suggested, since *K. terrigena* is not closely related to *K. ornithinolytica* and *K. planticola* on this basis [14]. Furthermore, in other bacterial clusters it has been considered that,

when the difference between 16S rRNA genes is greater than 5%, the clusters belong to different genera. It is therefore curious that, although the 16S rRNA gene sequences of *K. planticola* and *K. pneumoniae* are 97% identical, their separation into different genera was proposed [14]. Thus, 16S rRNA genes are not considered to be highly informative for the taxonomy of these groups, as discussed above.

Based on the sequences of *gyrB* genes, which code for the β-subunit of DNA gyrase, *Escherichia*, *Salmonella*, *Klebsiella*, *Enterobacter* and *Citrobacter* constitute a single group [12]. This is also observed with phylogenies derived from *infB*, which codes for initiation factor 2 [23]. Nevertheless, depending on the methodology used in the phylogenetic tree reconstructions with the *gyrB* sequences, the internal relationships of the genera within this group are different [12]. Still, based on the different trees generated with the *gyrB* sequences, it is clear that *K. terrigena* and *K. pneumoniae* [12] are two separate lineages, which justifies their placement into two independent genera, *Raoultella* and *Klebsiella*, respectively [14]. There are no *gyrB* sequences available from *K. planticola* and *K. ornithinolytica*. Evidence to support the inclusion of *K. planticola* in the new genus *Raoultella* may be derived from *gyrA* (which codes for the A gyrase unit) sequence analysis [50]. However, other sequences should be analyzed to confirm whether *K. terrigena* and *K. planticola* belong to the same genus. Two genetic groups have been recognized within *Klebsiella oxytoca* based on analyses of *rpoB*, 16S rRNA and β-lactamase genes and on PCR patterns produced by random repetitive primers [18].

Since phylogenies of a gene do not necessarily represent the phylogenies of a species [15], it has been recommended that about five gene sequences be analyzed when describing a species [53], along with the use of adequate reconstruction methods [53,59].

Population genetic structure of *Klebsiella*

If bacteria recombine extensively (therefore being panmitic) [39], any single part of the genome may not have a common evolutionary history with the rest. However, if recombination is limited and bacteria behave mostly clonally, then a few genes will reflect a substantial part of the genome. In order to minimize the effect of recombination, sequence analysis of approximately 11 genes has been recommended for bacteria such as *E. coli* [16], while in other cases a single gene seems to be representative of the majority of genes and gives phylogenies concordant to those of 16S rRNA genes [64].

Population genetic studies can be applied to detect genetic isolation, a biological concept useful in the recognition of new bacterial species [31,59,60]. Data derived from multilocus enzyme electrophoresis (MLEE) as well as from analyses of β-lactamase genes in comparison to housekeeping genes both in *K. oxytoca* [18] and in *K. pneumoniae* [22] indicate that the population structure in *Klebsiella* is clonal [11]. Furthermore, some chromosomally located β-lactamase genes seem to be of ancient origin, and do not recombine by lateral transfer [22]. Recombination among strains can be detected using DNA sequence data with incongruence length difference test (ILD) [6,32] or with split decomposition analysis [2,48], but these types of analyses have not been done for *Klebsiella*.

Klebsiella variicola, a model to define a bacterial species

The proposal of *K. variicola* as a new species was based mainly on sequence analysis of six genes belonging to several strains, including bacteria isolated in Mexico from plants (rice, maize, sugar cane and banana) and humans [50]. The genes used were: *rpoB*, *gyrA*, *infB*, *mdh* (which codes for malate dehydrogenase), *phoE* (which codes for a phosphate transporter) and *nifH* (which codes for nitrogenase reductase, involved in nitrogen fixation). These genes are scattered in enterobacterial chromosomes and correspond both to informative and metabolic genes as defined by Rivera et al. [49]. Informative genes are considered to be less prone to genetic recombination. Identical groupings were obtained irrespective of the gene analyzed. Phylogenetic trees were constructed using different methods. Figure 1 shows the phylogenetic position of *K. variicola* in relation to *K. pneumoniae* and to other species of *Klebsiella* based on *rpoB* sequences.

The fact that no sequences (of the analyzed genes) are shared between *K. variicola* and *K. pneumoniae* indicates that no exchange of chromosomal genes has occurred between these species; therefore, they seem to be genetically isolated [50]. Nevertheless, it seems possible that there is plasmid transference between these species (see below). Bacteria isolated from plants resembled *K. pneumoniae* according to biochemical tests designed for Enterobacteria [36]. It was found that around 8% of over 100 clinical strains identified as *Klebsiella pneumoniae* grouped with the strains from plants [50]. These clinical isolates together with plant isolates could be distinguished from *K. pneumoniae* because they were unable to use adonitol (a pentitol sugar, also called ribitol) as a carbon source. In *E. coli,* genes to catabolize adonitol are located at the same genetic locus as dulcitol (an
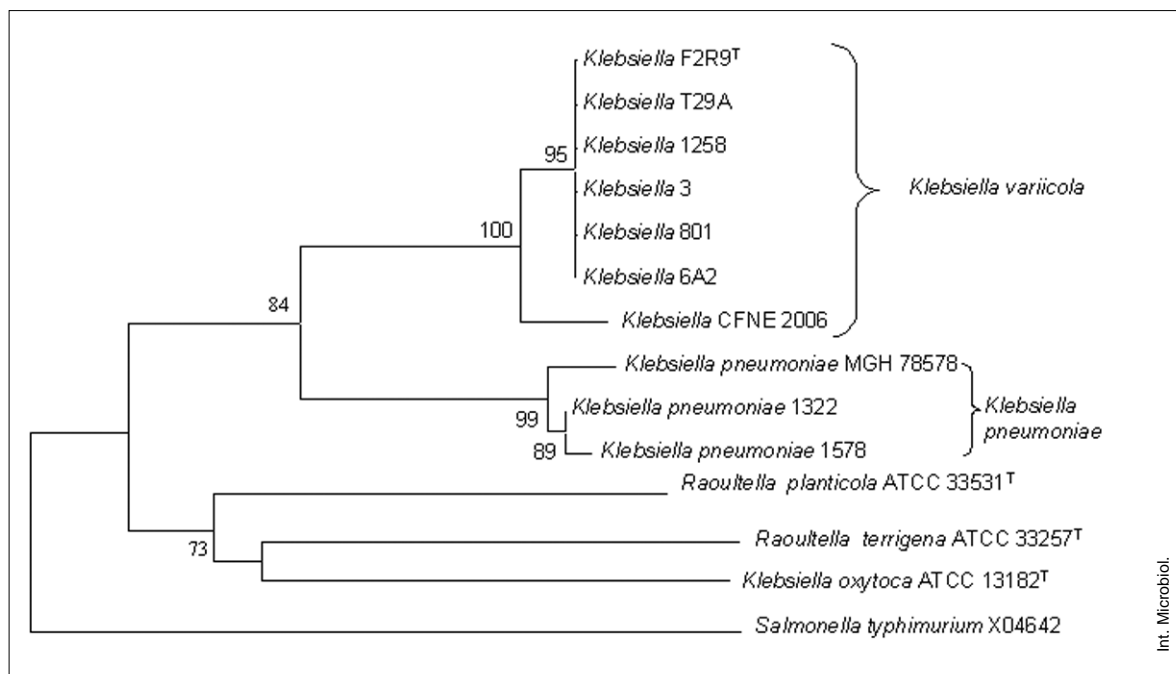
Fig. 1. Phylogenetic tree (Kimura 2-parameters) derived from *rpoB* partial gene sequences. All accession numbers are in [50] except CFNE 2006 (AY438584).

hexitol sugar, also called galactitol) catabolic genes, in a phenomenon called genotypic exclusion [34]. As such, when *E. coli* strains are dulcitol⁺, they are adonitol⁻. The catabolism of adonitol is a common characteristic of *K. pneumoniae* but *K. variicola* strains are adonitol⁻. We tested whether the latter were dulcitol⁺ but a majority were not capable of using this substrate (unpublished).

K. pneumoniae and *K. variicola* are separated by a considerable genetic distance, as determined from the analysis of metabolic enzyme patterns [36] and by their levels of DNA-DNA hybridization [50]. It is worth mentioning that when DNA-DNA hybridization results from *K. pneumoniae* and *K. variicola* were compared, very low levels (around 20-23%) were recorded with some *K. pneumoniae* strains , while with some *K. pneumoniae* from Mexico, higher values were seen (around 60-65%) (Table 3 in [50]). It remains unclear whether this is due to shared plasmids among sympatric *K. pneumoniae* and *K. variicola* strains.

All the *K. variicola* clinical or plant-associated isolates fix nitrogen, while only 10% of *K. pneumonia* strains have this capacity. *K. oxytoca* strains are also nitrogen-fixing [19]. Plasmids have been found in clinical isolates as well as in almost all of the bacteria isolated from plants [50]. Most *K. variicola* strains have plasmids of around 200 kb or less but they do not contain *nifH* genes, which are chromosomally located in *Klebsiella* species [50].

K. variicola strains are more susceptible than *K. pneumoniae* to the antibiotics spectinomycin and gentamycin. At 250 mg spectinomycin/l, all of the *K. variicola* strains we tested were sensitive to the antibiotic, compared to only 28% of *K. pneumoniae* strains. Regarding gentamycin, 82% of the *K. variicola* strains tested were sensitive to 10 mg antibiotic/l, whereas all *K. pneumoniae* strains were resistant [50]. In general, *K. variicola* is not resistant to high concentrations of carbenicillin (100 mg/l). Like *K. pneumoniae* strains, *K. variicola* strains are resistant this antibiotic (50 mg/l) but not in the presence of clavulanic acid [50], which inhibits β-lactamases. *K. variicola* strains are not resistant to cephalosporins, unlike many *K. pneumoniae* strains, since the latter have plasmids encoding extended-spectrum β-lactamases that could have been selected by antibiotics used in hospitals.

K. variicola includes also the group of *Klebsiella* described by Brisse and Verhoef as group III [5], which comprises 8% of the clinical bacteria considered as *K. pneumoniae* that were isolated from several European countries and Turkey. These were recognized as *K. variicola* by comparing *gyrA* sequences and because they were unable to use adonitol [50]. New data agree with this classification, as two other plant isolates were found to belong to group III, which also includes isolates from human feces obtained in Sweden and Japan [22]. Using the recently released *mdh* sequences (327 bp) from several group III strains [22], a new phylogenetic

tree was obtained that confirms the grouping of these strains with *K. variicola* (Fig. 2). The time of divergence of group III and *K. pneumoniae* (group I) has been estimated to be 6-28 million years [22]. We have calculated around 14 million years since the divergence of *K. variicola* and *K. pneumoniae* (unpublished).

The most-studied maize-associated *Klebsiella* strain, strain 342, also seems to belong to group III on the basis of ribotypes and random PCR patterns [13]. This strain could belong to *K. variicola* species because it has a *nifH* gene sequence identical to that of the *K. variicola* type strain [50], but the sequences of other genes are needed to clarify the taxonomic position of strain 342. The genome of this strain will soon be sequenced (E. Triplett, personal communication), which will clarify this issue and allow a very interesting comparison to the already sequenced genome of *K. pneumoniae* strain MGH 78578. It is worth mentioning that the latter is a non-nitrogen fixing bacterium.

Apparently, *K. variicola* and *K. pneumoniae* have different epidemiological dynamics. On the basis of their antibiotic resistance, nitrogen fixation capabilities, and virulence traits,

K. variicola seems to be better adapted to the environment than *K. pneumoniae*. *K. variicola* strains from plants could occasionally infect humans, while *K. pneumoniae* seems to have become more adapted to humans without much passage through the environment. In fact, the latter species is more resistant to antibiotics than *K. variicola* isolates, has lost (in a majority of strains) the capacity to fix nitrogen and is more virulent. Transmission from human to human seems more probable for *K. pneumoniae*. At any rate, it has been discussed that it is not advisable to introduce strains of *Klebsiella* into agricultural fields [35].

Klebsiella species nowadays

The species that are now considered within the *Klebsiella* genus are *K. pneumoniae* with its subspecies, *K. oxytoca* (with two subgroups) and *K. variicola*. The novel proposals of *Klebsiella* species not yet formally described with 16S rRNA gene sequences already available at the NCBI nucleotide data bases [http://www.ncbi.nlm.nih.gov/] may
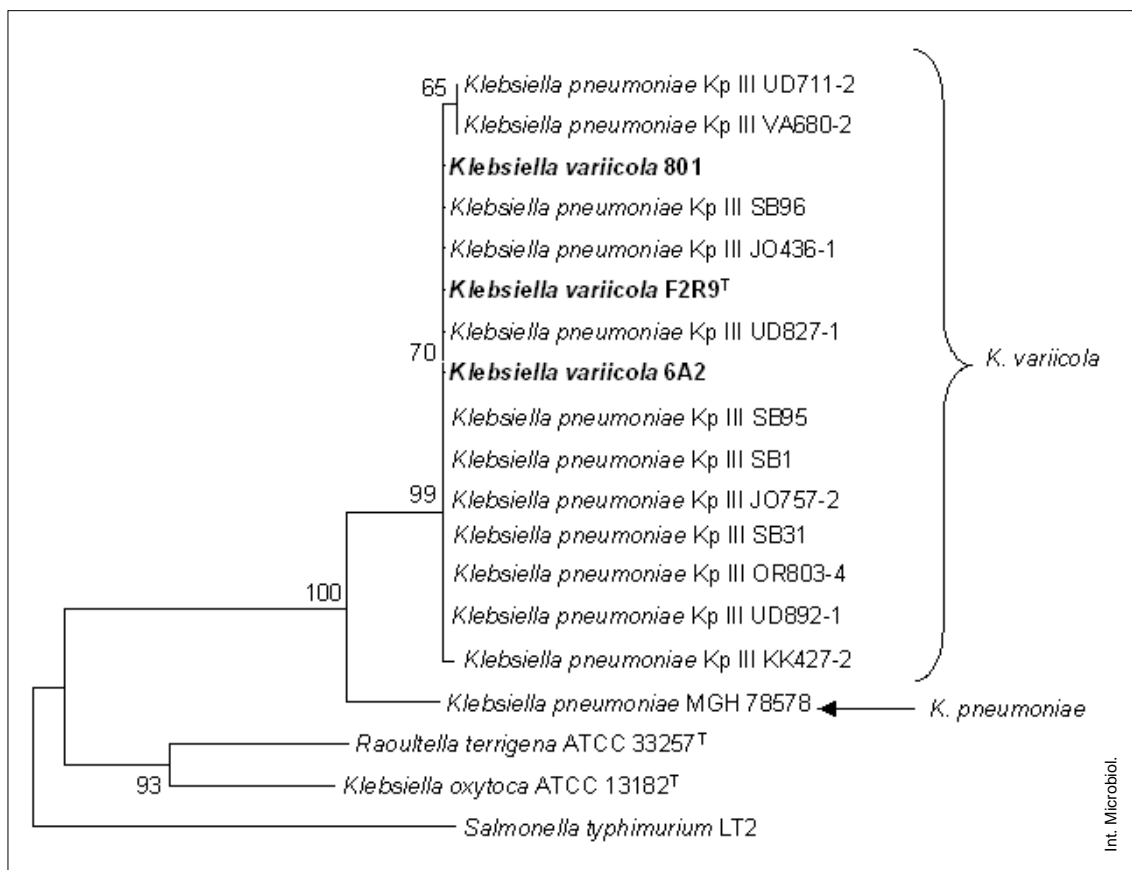

Fig. 2. Phylogenetic tree (Kimura 2-parameters) derived from *mdh* partial gene sequences.

enlarge this list in the future. *K. granulomatis* is an intracellular facultative pathogen that has not been cultivated in traditional media. There is evidence for considering it as a subspecies of *K. pneumoniae* [3,7].

Discussion

A polyphasic characterization to describe species has been recommended [51,58], but *Klebsiella* and other enterobacteria species were previously defined mainly according to phenotypic features [41]. Is phenotype-based taxonomy wrong? There are strain-to-strain differences in the ability to use specific substrates, and coincidences in substrate use is not a reliable argument for relatedness [4] since different species may retain this capacity from a common ancestor. Phenotypic characteristics seem to vary among related strains, probably by rapid loss of genetic information when not required or by acquisition via the lateral transfer of genes conferring an adaptive advantage for a particular condition [33,42]. In other bacteria, phenotypic data have been found to be in conflict with other taxonomic evaluations [52], raising questions as to the applicability of the polyphasic approach to bacterial systematics.

The information derived from gene sequences is certainly challenging current enterobacterial taxonomy. Comparative analyses of genomes of enterobacteria closely related to *Klebsiella*, namely *Escherichia coli* [61], are enlightening regarding what a species represents, i.e. a common (core) genome and "accessory" genetic material varying from strain to strain [31,61]. The conflicting position of *Pantoea* as a genus has been discussed [63]. *Shigella* species are lineages intermixed with *E. coli* [45]. Certainly we are facing a taxonomic revolution due to DNA sequence data. The same impact would not have been possible to achieve with phenotypic analysis or with DNA-DNA hybridization studies, which are limited to the number of strains analyzed and by their interpretation.

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¿Cómo están modificando los análisis de secuencias de genes la taxonomía bacteriana? El caso de *Klebsiella*

Resumen. Continuamente se cambian los nombres de las bacterias para describir más adecuadamente los grupos naturales (unidades de diversidad microbiana) y sus relaciones. Los problemas en la taxonomía de *Klebsiella* son ilustrativos y comunes a otros géneros bacterianos. Al igual que otras bacterias, las especies de *Klebsiella* fueron descritas hace mucho tiempo, cuando los métodos para identificar y clasificar bacterias eran limitados. Diferentes enfoques moleculares, desarrollados sólo recientemente, han conducido en muchos casos a revisiones taxonómicas o a propuestas sólidas de nuevas especies. [**Int Microbiol** 2004; 7(4):261-268]

Como as análises de sequencias dos genes estão modificando a taxonomia bacteriana? O caso de *Klebsiella*

Resumo. Continuadamente são mudados os nomes das bactérias para se descrever mais adequadamente os grupos naturais (unidades da diversidade microbiana) e suas reais relações. Os problemas na taxonomia de *Klebsiella* são ilustrativos e comuns a outros gêneros bacterianos. Semelhantemente a outras bactérias, há muito tempo se conhece *Klebsiella* spp., mesmo quando as possibilidades de se identificar e classificar bactérias eram limitadas. As metodologías moleculares para identificar e classificar bactérias desenvolvidas só recentemente têm conduzido, em muitos casos, a revisões taxonômicas ou a propostas firmes de novas espécies. [**Int Microbiol** 2004; 7(4):261-268]