

# IT'S COMPLICATED: DIFFICULT RELATIONSHIPS BETWEEN MICROBIAL ECOLOGY AND TAXONOMY

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## Abstract

Taxonomy is a branch of the sciences that is focused on the classification of organisms. A crucial problem for microbial taxonomy is elaboration of a coherent definition of bacterial species. With the aid of *Bacillus cereus* sensu lato, we discuss the different ideas for this definition and demonstrate the role of ecology and the ecological niche occupied by the bacterium in its evolution. We conclude that direct application of an actual definition of a bacterial species is not always possible.

**Keywords:** *Bacillus cereus* sensu lato, natural selection, species, the phylo-phenetic definition of species

## Introduction

Taxonomy is a branch of science that encompasses the identification of particular organisms as well as their classification with suitable nomenclature and descriptive properties. Among distinct approaches important in taxonomy, especially evolutionary taxonomy based on Darwin's findings and post-Darwinian classification, phenetic taxonomy (that requires comparison of overall similarities) and phylogenetic conception (based on features derived from common ancestors) are widely discussed and used in scientific practice (Cohan, 2002b; Małek et al, 2005). Ecology is focused on any interactions between different organisms (including intraspecific and interspecific relations) or between organisms and their environment. Those relations may have both positive and negative impacts on the studied life forms, but if they are not neutral, they will play an important role in their evolution (Bartoszewicz, 2018). Consequently, we cannot analyze that evolution without paying attention to the habitats and ecological niches occupied by each particular species, because no organism is completely isolated from other life forms and its natural environment.

Naturally, the entire relationship between ecology and taxonomy has been widely studied. However, most findings are based on animals and plants. In opposition to them, knowledge about microorganisms and their evolution in

the context of their ecology is not so wide. Moreover, we still do not have a precise definition of bacterial species, and the criteria used for identification of microorganisms are often far from being perfect. Thus, the first step on the way to understanding the interesting relation between ecology and other sciences in microbiology must start with elaboration of a coherent definition of the bacterial species (Konstantinidis and Tiedje, 2005; Konstantinidis et al., 2006; Wiedenbeck and Cohan, 2011). So we have decided to discuss different approaches to this problem and the criteria used for precise identification and differentiation between microbes by the aid of model organisms.

## Why does the definition of a eukaryotic species not fit bacteria?

For several years, scientists have tried to elaborate a coherent definition of species as it is fundamental for taxonomy and systematics. Actually, following Mayr's ideas, species are defined as a basic unit of biological classification which groups genetically-related individuals capable of producing fertile offspring as a result of sexual reproduction. Organisms classified into different species should be also reproductively isolated from others, which naturally do not preserve formation of sterile hybrids. Following these criteria, we can conclude that organisms from one species usually occupy similar ecological niches (but their range depends on their ecological tolerance, which is different for distinct species) and should originate from a common ancestor. Such a definition reflects the evolutionary traits and fits very well with the natural systematics. Naturally, as in most cases with living organisms involved, here we can also expect some doubts and challenges. For example, it is difficult to assess where the border of a species is if a ring species is found. One of the popular examples (however also recently discussed) are herring gulls (*Larus argentatus* and *Larus fuscus*) – for example, by Liebers et al. (2004) or by Martins and Packert (2007). Nevertheless, ring species are found to be a bridge between the micro- and speciation process (Irwin et al., 2001). They form distinct populations which do not interbreed, but they are connected by a geographical ring of populations that may interbreed. Next, common shrews (*Sorex araneus*) are represented by numerous chromosomal races (races with different karyotypes formed in the process of Robertsonian fusions of chromosomes). However, representatives of different races are able to have offspring whose fertility is reduced (but often not blocked) due to severe complications in meiosis (eg during the conjugation of chromosomes, prophase I).

Unlike the research focused on eukaryotes, studies on bacteria have a much shorter history. First reports on the existence of microscopic forms, yet unknown, were provided by Antoine van Leeuwenhoek in the seventeenth century. The next microscopic observations, description of bacterial shapes, and even Pasteur's theory of fermentation caused by microbes were only initial studies. Finally, Robert Koch formed and elaborated a method for isolation of pure bacterial cultures. This finding enabled precise description of the properties of

known microbes and, with Koch's postulates, facilitated differentiation between commensal and pathogenic bacteria. However, still, the describing of bacterial species was a case of artificial taxonomy because it reflected only phenotypic and/or ecological similarities.

Even later, after Mayr published his definition of the species, bacterial taxonomy was based on different criteria (Mayr, 1942). The most important problem was related to the lack of sexual reproduction among prokaryotes (Cohan, 2002b). Bacteria proliferate by cell division, and as a result of this process, two daughter cells are formed. Apart from spontaneous mutations that may occur during chromosome replication, those daughter cells are identical, so bacterial reproduction leads to the formation of a population of clonal cells. Next, the diversity observed among microbes is much higher than among eukaryotes. A high polymorphism rate is an effect of multiple factors. First of all, bacteria are haploids, so mutations (besides neutral and silent) are visible in their phenotype. The lack of nuclear membrane makes bacterial chromosomes much more exposed to different damaging factors, like bacteriophages or free radicals. This, combined with the weaker repair capabilities (weak correctional activity of bacterial DNA polymerase) and lower fidelity of DNA replication, results in high diversity, even among a group of closely related microorganisms (Bartoszewicz, 2018). We also should be aware that most bacteria (especially at optimal environmental conditions) multiply very rapidly and quickly, often with the generation time below half an hour. This leads to successive generations in a short period of time. Even if the mutation rate is constant, with each of the numerous generations the chance of mutation also increases. Next, which remains in connection with ecology, bacteria, even pertaining to one species, occupy different niches. Even species typically associated with animals, like *Escherichia coli* (its primary habitat is the colon, a part of the large intestine) can be found in soil samples or as a form of municipal pollution in water. Finally, this bacterium can be found in food products as a consequence of poor hygiene standards and selected isolates may lead to the development of infections (eg enterohaemorrhagic isolates, EHEC). Such a wide range of potentially isolated habitats requires different adaptations, but it results in independent accumulation of mutations and polymorphism (Cohan, 2002a). As mentioned before, bacteria are not able to reproduce by a sexual process. Instead, they have developed a few parasexual processes, which allow them to exchange their genetic information. Conjugation, the process of transferring (usually) plasmid DNA between a donor (a cell that has already replicated its plasmid or replicates it during the conjugation event) and a recipient (a cell that obtains DNA from the donor), results in propagation of some genetically encoded features in the bacterial population. Some of these features might be evolutionarily beneficial, like antibiotic resistance for clinical isolates. Another kind of horizontal gene transfer (HGT) is transformation, where bacteria obtain DNA from the environment and may incorporate such a fragment into its own chromosome and express the genetic information. The effect of this process, described for streptococci, is phenotype change, from apathogenic to pathogenic. Finally, transduction, another case of HGT but involving bacteriophages, may cause formation of a stable recombinant form.

What is interesting, the kind of environment is important here. For example, conjugation is much more frequent and effective in habitats rich in nutrients, probably because of easier to achieve higher concentrations of microbes. Thus, food matrices may be endangered by this phenomenon. Another surprising fact that complicates the taxonomy of microbes is the need to establish a pure culture prior to the describing of a new species. The percentage of viable but non-culturable microorganisms (VBNC) is not precisely established, but we could expect their high frequency, especially in environments where mineral compounds dominate, and among autotrophic bacteria (Oliver, 2005).

Naturally, we completely omit another unique form placed on the border between life forms and complex chemical particles, viruses. On the one hand, they do not behave like living organisms outside of its host, so there is no replication, metabolism, or motility observed in the environment. On the other hand, while present in cells, they are replicated and released; moreover they undergo phenomenon typical to organisms, like inheritance of their properties, mutation, or evolution. What is more, different viruses may have distinct genetic material (dsDNA, ssDNA, ssRNA, dsRNA) with unusual organization (segmented, circular, linear, covalently closed). In the present paper, the problems of taxonomy of viruses will therefore not be included.

## Phylo-phenetic definition of bacterial species

According to the facts provided above, Mayr's definition does not suit the prokaryotes. However, we could expect that similar processes, like in the case of eukaryotes, will lead to speciation. Speciation is in general a result of some kind of isolation and subsequent accumulation of distinctive features. So, the gene flow between representatives of different species should be prohibited or at least reduced. Next, natural selection should favor beneficial properties (in the context of actual environmental conditions) and eliminate unfavorable features. Here we meet an important correlation between ecology and evolution. Bacteria with wide distribution in the environment are often more diverse. This is a result of the need to adapt to different conditions present in the natural state. Complex niches (like soil) with its different structure and nutrients, require the ability for multiple metabolic processes necessary for obtaining energy from distinct substrates. Typical pathogens with a narrow range of hosts must be, in turn, adapted to one niche. Their requirements include mechanisms of avoiding host immune system mechanisms, rapid proliferation, and resistance to the most common antibacterial therapeutic agents. This may be observed in the case of *Klebsiella pneumoniae*, which is quite common, but the 'hospital environment' pressures resulted in selection for the resistant isolates and finally development of *K. pneumoniae* NDM-1 (also known as *K. pneumoniae* New Delhi strain) producing metallo- $\beta$ -lactamases and being resistant to plenty of antibiotics used in therapy (Khan et al. 2017).

All these facts were considered in order to elaborate a suitable definition of a bacterial species. At the early beginning of microbiology, based on bacterial

shape, colony morphology, virulence, and substantial biochemical properties, an artificial taxonomy was constructed. It was useful in medicine and veterinary research, however this approach did not reflect evolutionary relationships between organisms. Next, deeper and more detailed biochemical analyses became important and they enabled a first attempt to reconstruct phylogeny on the basis of similarities that might be genetically encoded. Moreover, numerical methods could be applied for biochemical data. However, the most important data were provided by molecular techniques. Similarities in G+C content, total DNA-DNA hybridization (DDH) reflecting the overall similarities between genomes of compared bacteria, gained importance. Next, sequencing of housekeeping genes (in MLST technique, multi-locus sequence typing) or comparison of nucleotide sequences of ribosomal DNA (eg 16S rRNA gene) depicted provided data that were suitable for reconstruction of phylogeny. Finally, the recent approach is based on the comparison of complete genomes. This method is getting cheaper and the special equipment necessary for this procedure becoming more accessible. Unfortunately, even recent molecular techniques are not ideal. 16S rRNA genes may be present in several copies in the bacterial chromosome and there is no guarantee of their identity in one isolate. Next, conservative sites display a low level of variability and only short fragments represent hyper-variable sites, so the comparison of closely related microbes could be difficult and hard to note. In turn, new generation sequencing, a powerful tool for the assessment of complete genomes, detects all the variability and provides huge pieces of data that might be difficult to interpret.

Summing up all these troubles and limitations, a phylo-phenetic concept of species in bacteriology was proposed (Rossello-Mora and Amann, 2001). It assumes that bacteria pertaining to one species must have at least 97% of identity in the 16S rRNA gene sequence, not less than 70% of similarity in the DNA-DNA hybridization, and one or more phenotypic distinctive features. This definition, accepted by most microbiologists, meets some restrictions, especially in relation to groups of related bacteria that differ mainly in their phenotype. Thus, we decided to verify how a phylo-phenetic definition of bacterial species suits *Bacillus cereus* sensu lato rods.

## What is *Bacillus cereus* sensu lato?

*B. cereus* sensu lato (often named the *B. cereus* group) comprises several bacterial taxa, which are common in the natural environment and play important roles in ecology, medicine, and in the dairy industry. The best-known representative of the whole group is *Bacillus cereus* (also named *B. cereus* sensu stricto). This bacterium is found in soil, however it easily penetrates into the food processing chain, mainly on dairy farms (Bartoszewicz and Czyżewska, 2017). Consequently, it is commonly found in raw and pasteurized milk, salads, spices, pasta, and vegetables. Unfortunately, *B. cereus* is able to produce several food-poisoning toxins that create a health hazard to consumers. Among the toxins, hemolytic enterotoxin (HBL), non-hemolytic enterotoxin (Nhe), or cytotoxin K

(CytK) are dangerous even when only a small piece of contaminated food undergoes consumption because they are produced in the small intestine by vegetative forms of the mentioned bacteria. After reaching sufficient concentrations, they interact with the intestinal epithelium, leading to the development of symptoms of diarrheal syndrome. In turn, cereulide, a dodecadepsipeptide ring structure toxin (with important resemblances to the ionophore antibiotic, valinomycin) is produced in food prior to its consumption (Bartoszewicz and Czyżewska, 2017; Kroteń et al. 2010). It is a quite stable peptide, so even heat-treatment and denaturing chemical factors do not inactivate cereulide. Its intoxication causes disturbance of ATP synthesis in mitochondria, which leads to acute liver and kidney failure, which might be fatal. Like the other representatives of *B. cereus sensu lato*, this species is able to form spores (called also endospores) which are resistant to UV light, drought, lack of nutrients, as well as high temperature or freezing. Dormant spores may survive in food processing lines and germinate even after several years of unfavorable environmental conditions. Spores can be easily dispersed by the air (wind) or water. For many years it was thought that spores are common in the natural environment, but vegetative cells with metabolic activity are typical only to restricted environments, like the digestive tracts of invertebrates or mammals. Since recent findings have proved the presence of vegetative cells in plant material (eg cabbage) or in the freshwater of Poland, this assumption has had to be revised. Consequently, if vegetative cells are more common than previously thought, they may undergo horizontal gene transfer and interactions with the environment. Moreover, natural selection in wild-type habitats could favor not only strains forming extremely resistant spores but also those with cells able to use nutrients present in the environment.

Another important representative of the mentioned group is *Bacillus anthracis*, which causes anthrax, a dangerous disease of mammals including humans. This species synthesizes a unique capsule and toxins responsible for the development of the symptoms. Its virulence is directly linked to two mega plasmids, pXO1 and pXO2. It is puzzling that similar plasmids (but often devoid of the pathogenicity island) have been found in numerous isolates of *B. cereus*. The next species, *Bacillus thuringiensis* is known due to its insecticidal activities. *B. thuringiensis* owes its properties mainly to *cry* genes located on plasmids that potentially could be mobile and undergo horizontal transfer. Finally, psychrotolerant *B. mycoides* with rhizoidal colonies on solid media and *B. weihenstephanensis* (recently recognized only as a variant of *B. mycoides*) are able to proliferate at low temperatures (4-7°C), but their multiplication completely stops above 43°C. Other species classified into the *B. cereus sensu lato* (eg *B. toyonensis*, *B. cytotoxicus*, *B. wiedmannii*) have little importance or are not commonly accepted and described on the basis of digital DNA-DNA hybridization (like *B. manliponensis*, *B. bingmayongensis*, *B. gaemokensis*). For more details, please see the review by Bartoszewicz and Czyżewska (2017).

## Phylo-phenetic species definition vs. *Bacillus cereus* group

In order to assess the correctness of the phylo-phenetic species definition, we should follow all the criteria and try to apply them for the mentioned bacteria. So, the first point is the similarity level of the 16S rRNA gene sequence. For bacteria in one species, we expect values >97%, while members of one genus should display at least 95% identity. Numerous studies using sequencing of this gene, as well as alignments of sequences available in public databases (eg NCBI, National Centre for Biotechnology Information) clearly show that *B. cereus* sensu lato has less than 1% of polymorphic sites in the 16S rRNA gene. This directly leads to the conclusion that we are dealing with one species. However, there is an important rule. If the similarity of the 16S rRNA gene sequence is below the threshold value, this is evidence for a distinct species. However, values above 97% should be interpreted as the lack of evidence for different species, not as evidence for one species.

The next criterion in modern taxonomy is the level of similarity in DDH. This method is based on spectrophotometrical measurement of the hybridization of DNA isolated from two compared isolates. This method is informative; however, recently its variant, dDDH (digital DDH) has become more common and useful. If strains display more than 70% similarity, they should be assigned to one species. Consequently, lower values confirm two different species. However, even from our studies performed in 2012-2016, we found two isolates representing *B. cereus* and *B. thuringiensis* that shared 76% similarity in DDH (which indicates one species) and surprisingly we also found isolates from distinct sources (soil, milk, freshwater) that were phenotypically and genetically identified as *B. cereus* (case 1) and *B. weihenstephanensis* (case 2), though they showed only 37% (case 1) and 43% (case 2) similarity, respectively. Moreover, dDDH is believed to be too sensitive, which could result in description of a new species that in fact does not yet exist.

Finally, as mentioned above, bacteria belonging to one species must have at least one, common among them but distinctive from others, phenotypic property. This is a seemingly simple situation here. For example, *B. cereus* differs from *B. thuringiensis* by the lack of parasporal crystal inclusions. They are quite easy to find under phase-contrast microscopy and their presence clearly indicates that we are dealing with *B. thuringiensis*. However, one can ask what would happen, if during rapid proliferation this property is lost in the process called curing? Without plasmids containing *cry* genes, it is impossible to synthesize crystal proteins and *B. thuringiensis* becomes indistinguishable from *B. cereus*. Another problem was shown by Bartoszewicz et al. (2009). Authors isolated *B. thuringiensis* from soil (NE Poland) that shared psychrotolerant characteristics of *B. weihenstephanensis*. A proposed explanation of this fact is horizontal transfer of the *cry* genes from *B. thuringiensis* to recipient cells of *B. weihenstephanensis*. However, the taxonomic affiliation of these strains is confusing and problematic. They represent features typical for two different taxa and meet the criteria enabling identification of them as both *B. thuringiensis* and *B. weihenstephanensis*. Further experiments with conjugation proved that HGT may occur within *B. cereus* sensu lato leading to the formation of cold-adapted strains with protein

crystals in the described way, but the efficiency of this process is extremely low (Bartoszewicz, unpublished data). Another example is *B. mycoides* and *B. weihenstephanensis*, which both, in fact, are psychrotolerant. In this context, the only way for differentiating between them is to compare their colonies on solid media: *B. mycoides* forms rhizoidal forms in opposition to the typically round colonies of *B. weihenstephanensis*. Finally, typical properties of *B. anthracis* are linked to its virulence associated with two large plasmids: pXO1 and pXO2. Genes from these extrachromosomal DNA encode toxins and capsule, both necessary for causing symptoms of anthrax. Actually, we know that the same type of plasmids (called pXO1-like and pXO2-like) are common in the entire *B. cereus* group and do not always result in virulence (Bartoszewicz and Marjańska, 2017). Apart from distribution of the pXO1 and pXO2-like plasmids, *B. anthracis* has its own properties, like susceptibility to penicillin or lack of motility. Moreover, probably because of the need to adapt to a narrow niche and the activity of purifying selection, this pathogen is much more homogenous and may represent an independent evolutionary line within the group.

Thus, several authors have suggested that species within *B. cereus* group may only play a role in applied microbiology and be useful in clinical diagnostics due to the properties traditionally associated with particular taxa. Nevertheless, an approach that recommends remaining on the bacterial group level or even genus level, without distinguishing individual species, can definitely reflect our current knowledge better than strictly following and applying the phylogenetic definition of species. The enormous role of environmental pressures affects bacterial characteristics, including their stability and diversity, proliferation rate, occurrence of mutations, specific adaptations, and the frequency and efficiency of horizontal gene transfer.

## Conclusion

Summing up these facts, we have to say that the current criteria for bacterial species play their role for most microbes; however in some cases, they are still problematic. Thus, it is necessary to elaborate a brand-new approach for bacterial taxonomy: an approach that includes the difference of microorganisms, their high rate of polymorphism, problems associated with horizontal gene transfer and data obtained from well-performed studies including the entire genome sequencing. And until that moment, we have to remain aware that the systematics and taxonomy of bacteria are partly artificial and based on arbitrarily set criteria. An approach based on ecotypes (ecological types of bacteria within a species, eg pathotypes, thermotypes) much better reflect their characteristics and effects of natural selection, so they should be also considered in further bacterial taxonomy.

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