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Ecology and evolution of bacterial microdiversity

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Abstract

Using high resolution molecular fingerprinting techniques like random amplification of polymorphic DNA, repetitive extragenic palindromic PCR and multilocus enzyme electrophoresis, a high bacterial diversity below the species and subspecies level (microdiversity) is revealed. It became apparent that bacteria of a certain species living in close association with different plants either as associated rhizosphere bacteria or as plant pathogens or symbiotic organisms, typically reflect this relationship in their genetic relatedness. The strain composition within a population of soil bacterial species at a given field site, which can be identified by these high resolution fingerprinting techniques, was markedly influenced by soil management and soil features. The observed bacterial microdiversity reflected the conditions of the habitat, which select for better adapted forms. In addition, influences of spatial separation on specific groupings of bacteria were found, which argue for the occurrence of isolated microevolution. In this review, examples are presented of bacterial microdiversity as influenced by different ecological factors, with the main emphasis on bacteria from the natural environment. In addition, information available from some of the first complete genome sequences of bacteria (*Helicobacter pylori* and *Escherichia coli*) was used to highlight possible mechanisms of molecular evolution through which mutations are created; these include mutator enzymes. Definitions of bacterial species and subspecies ranks are discussed in the light of detailed information from whole genome typing approaches.

Keywords: Microdiversity; Microbial ecology; Microbial evolution; Whole genome analysis; *Burkolderia*; *Paenibacillus*; *Ochrobactrum*; *Pseudomonas*; *Frankia*; *Rhizobium*; *Helicobacter*; *Escherichia coli*; *Stenotrophomonas*; *Xanthomonas*; *Ralstonia*

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1. Introduction

With the rise of molecular genetic tools in microbial ecology, it became apparent that we know only a very small part of the diversity in the microbial world. Most of this unexplored microbial diversity seems to be hiding in the large amount of yet uncultured bacteria. For example, using the rDNA-directed approach of dissecting bacterial communities by amplifying the 16S rDNA (*rrs*) gene from soil or sediment samples by polymerase chain reaction (PCR), and studying the diversity of the acquired *rrs* sequences, almost exclusively new sequences were isolated. These are only related to a limited degree to those from well-studied bacteria in culture collections. Frequently occurring yet uncultured bacteria became visible microscopically by using fluorescently labelled rRNA-directed oligonucleotide probes [1]. As a bacterial species is composed of different geno- and phenotypes, there is even more structural and functional diversity below the species level, which we call microdiversity. Since genetic fingerprint techniques based on the whole genomes of microbes and high resolution phenotyping methods are widely applied, a new level of bacterial diversity can be studied. Detailed molecular genetic information may help us to understand evolution of the functionality of microbes in their particular environment, creating new genetic variants below the species level. The selection of newly acquired functions may give us the key to understanding the functional diversity of microbial communities and ultimately of ecosystem function as a whole.

In this review, we present information on the microdiversity of bacterial populations with special reference to bacteria from the natural environment using high resolution genetic fingerprinting techniques and the knowledge arising from whole genome sequences. Basic questions about the evolution and ecology of bacterial diversity can be tackled on the basis of this new quality of information. This also prompted us to evaluate the present use of taxonomic terms below the species level. We do not present data dealing with intraclonal diversity (phase variation) which can also be considered as a rapid adaptation to different environments [2].

2. Bacterial taxonomy at and below the species level definitions, problems and new approaches

A prerequisite of studies on microbial diversity is that they are performed at distinct levels of resolution on clearly defined operational taxonomic units (OTUs). This requires clear and, as far as scientifically possible, universally valid definitions of the respective taxonomic ranks. Definitions of taxonomic ranks categorizing evolving organisms are a priori arbitrary.

Most research on microbial diversity is conducted at or below the species level. The term for diversity used should indicate the taxonomic level within which diversity is assessed. For example, intra(sub)species diversity implies that the upper level of resolution is a distinct (sub)species. We use the term 'bacterial microdiversity' if diversity of distinct pheno- or genotypic groups is analyzed, but the taxonomic level is not yet clear. In the following, we introduce the relevant taxonomic ranks: species, subspecies, infrasubspecies, strain and geno- and phenotype-based categories, discuss briefly immanent and associated difficulties, and present developments that may be helpful to overcome these.

2.1. Species

The basic official taxonomic group in modern bacterial systematics is the species [3], but there are presently no common official rules for the classification of bacteria. Bacterial systematics is inconsistent and sometimes confusing because different concepts (taxonomic, phylogenetic and biological approaches) have been and still are applied simultaneously to delineate species.

Most bacteriologists accept that bacterial species should be defined by a polyphasic taxonomic approach [4] integrating results from numerical pheno- and genotyping and rRNA gene homology studies. In the currently applied polyphasic taxonomic concept, a species is delineated from others and can be defined if pheno- and genotyping analyses differentiate the investigated group of strains from related species in consensus, if diagnostic phenotypic characters for the new species are found [3,5], and type strains are deposited in culture collections. Priority is given to the results from DNA/DNA hybridization because comparing 16S rDNA (*rrs*) sequences provides only limited resolution (see below; [5]). DNA relatedness should be less than 60–70% to differentiate two species. At values between 60 and 70% DNA reassociation, differences in thermal stability between homo- and heterologous DNA hybrids should be higher than 5°C [3,5]. Analyses of *rrs* gene similarity may substitute for DNA/DNA hybridization studies in the description of new species, if the (almost) complete *rrs* sequences differ by more than 3% [5]. Several recent species descriptions made use of this more convenient approach [6,7].

Inconsistencies in systematics of bacteria arise from the simultaneous application of different species concepts (see above). A serious obstacle to a unified species concept is subjective consideration of practical usefulness for species definition. Particularly in the group of human and animal pathogens, many species are delineated primarily on phenotypic traits such as host range preference and pathogenicity. *Brucella* species, for example, show interspecific DNA relatedness above 98%. Verger et al. [8] proposed therefore a monospecies (*Brucella melitensis*) concept comprising six biovars and respective biotypes for *Brucella* strains, but this concept has still little acceptance in the scientific community working on microbial pathogens [9].

Culture independent studies on heterogeneity of *rrs* gene sequences from environmental sources suggest that many hitherto unknown bacterial lineages exist which are different from described species [10]. One can extrapolate from *rrs* gene dissimilarity that most of them constitute new species. An increasing number of sequences, genomospecies and candidatus (see below) cannot be given official species status because they cannot yet be studied using culture dependent phenotyping approaches and DNA/DNA hybridization, and necessary type strains are not available as references. If the phylogenetic position of a sequence is found within a distinct physiological group, this may, in combination with other techniques, lead to the development of suitable cultivation conditions for the respective organism [11].

2.2. Subspecies

Subspecies is the lowest phylogenetically supported taxonomic rank that has official standing in nomenclature. Subspecies designation can be given to genetically determined clusters of strains or to groups of strains exhibiting minor but consistent phenotypic variation within a species [12,13], as it is currently defined (see above). Type strains must be available from culture collections. Subspecies have been created in many genera. *Bacillus subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii* may serve as an example. They show DNA relatedness of 58–69%, and differ in cell wall composition [14]. The clinically relevant *Staphylococcus hominis* subsp. *novobiosepticus* and *S. hominis* subsp. *hominis* are genetically closely related but significantly divergent, and differ in antibiotic resistances and conversion of certain substrates [15].

For a better resolution of diversity below the species level, we recommend that more use is made of the subspecies concept. However, the lower delineation level should be defined. This is possible using genetic distances as provided by genotype fingerprinting techniques which are being calibrated on other approaches such as DNA/DNA hybridization (see Section 2.5).

2.3. Strain

Ideally, a strain is a clonally proliferating organism. Its descendants should be genetically identical. However, organisms are subject to mutation and DNA transfer producing genetic diversity, and new phenotypes may be selected (see Section 3). The chance for genomic variation within a bacterial clone is not negligible [16], and therefore the assessment of strain identity and detection of diversification can be delicate. Problems can arise, if differentiating phenotype characters are not found, if phenotypes are variable in different growth phases, or if genotypes of strains exhibiting different colony morphology are variable even under standardized conditions. Repeated cultivation of a strain under artificial (laboratory) conditions may cause a shift in the genotype (e.g. [17]).

The resolution limits of the methods used determine the quality and quantity of observed mutation events. Several mutations in non-coding DNA regions that do not affect the phenotype and are not detected using low resolution approaches may be revealed using highly sensitive broad-range genotyping methods (see Section 2.5). A single mutation in expressed loci, such as *hrp* or *vir* genes in strains of two *Pseudomonas syringae* pathovars, however, can alter the phenotype [18,19] but will genotypically only be detected by specifically targeted probes or genomic sequencing.

High resolution genotyping methods should be applied judiciously in combination with phenotyping approaches to assess strain identity. Genetic stability and authenticity of a strain may be maintained only under conditions which, as far as possible, avoid mutation and selection. References, such as well-documented lyophilized strains of culture collections and nucleic acid sequence entries in databanks, are the basis of taxonomic research and studies on diversity.

2.4. Other taxonomic definitions below the species level

2.4.1. Genotype-based terms

In this chapter, we report on frequently used genotype-based terms (i) for non-cultivated OTUs that are most probably new species and (ii) for cultivated OTUs which group at or below the species level, but which lack the characters required for species description.

Candidatus status is provisional and can be given to prokaryotic entities for which more than a mere DNA sequence is available, but for which characteristics required for a description according to the International Code of Nomenclature of Bacteria are lacking [20]. Candidatus category can be given to incompletely described prokaryotes for which genome-derived data indicate that they are new species (e.g. if *rrs* sequences retrieved from environmental DNA diverge from known *rrs* sequences by more than 3%) and for which additional information on structural, metabolic or reproductive features is presented. Many candidatus can be found e.g. in the genera *Helicobacter*, *Phytoplasma* and *Mycoplasma*. '*Candidatus* Endobugula sertula' [21] and '*Candidatus* Parachlamydia acanthamoebae' [10] are examples, where the development of labelled specific *rrs* gene targeted oligonucleotides and subsequent in situ hybridization with the respective probes confirmed the origin of the sequences, indicated that the organisms were present in the samples, and gave information on their cell shape.

Genomospecies, genospecies, genomic species or genetic species are groups of cultivated organisms for which data from nucleic acid comparisons indicate that they constitute new species (see above), but for which no distinguishing phenotypic properties have yet been found. Upon

identification of such characters, they can be defined and named as species [3,13]. In order to unify and simplify the concept, only the term genomospecies should be maintained.

Numerous unnamed genomospecies have been described, e.g. within the genus *Pseudomonas* [22]. *P. syringae* is an example of an apparently heterogeneous species in which genomospecies are delineated (see Section 4.1.4). Three out of five former *Burkholderia cepacia* genomospecies have meanwhile been given official species status because differentiating phenotypic characters were identified [23].

Genomovar is frequently misinterpreted as genomospecies. Genomovars are cultivated strains or groups of strains that constitute genotypic entities below or at the subspecies level, but differential phenotypic characters required for categorization as subspecies are lacking. Many OTUs hitherto described as genomovars are in fact genomospecies. Strains exhibiting distinct genotype fingerprinting profiles, e.g. within *Stenotrophomonas maltophilia* (see Section 4.1.3), may be regarded as genomovars. Song et al. [24] delineated two genomovars within *Azoarcus tolulyticus* and *Azoarcus toluclasticus*, respectively. In spite of very high intergroup *rrs* gene similarity of over 99%, relatively low DNA reannealing of 49–65% and 47–55% indicates that each of the genomovars might as well represent genomospecies.

2.4.2. Phenotype-based terms

The following terms are used for single (or groups of) strains that were hitherto distinguished only by particular phenotypic traits, and are grouped therefore at the infrasubspecific, i.e. below the subspecies, level. Such ranks have no official standing in nomenclature, but often have great practical usefulness [12]. This classification was designed to meet practical needs of distinct disciplines. It is subjective, must be regarded as provisional, and should be replaced by a classification system which is based on a universally applicable and valid parameter for the delineation of OTUs below the species level, e.g. on DNA similarity, as assessed using genotype fingerprinting methods (see Section 2.5).

Pathovar (pv.) is used for a group of strains exhibiting pathogenic properties for certain hosts. Pathovars of species are found in a variety of different genera, e.g. *Xanthomonas*, *Pseudomonas*, *Burkholderia*, *Erwinia* or *Ralstonia*. Virulent and avirulent races of a given pathovar induce necrosis differentially on sets of plant cultivars within agronomic species [25]. A race can comprise several biovars (see also below) which are distinguished by substrate utilization or host association. In a study on *Ralstonia* (formerly *Pseudomonas) solanacearum* race 1 strains, genomic fingerprinting (RC-pulsed field gel electrophoresis (PFGE), repetitive extragenic palindromic PCR (rep-PCR)) revealed genetic groups within that race. Most of them were clustered in agreement with bacteriocin and biovar groups [26]. Rep-PCR, amplified (DNA) fragment length polymorphism (AFLP) and ribosomal spacer analyses were effective in determining intrapathovar diversity of xanthomonads and *P. syringae* [17,27,28]. The inconsistency of the pathovar and biovar systems is discussed below.

Biovar (bv.) is used for particular strains of a species displaying differential phenotypes, special biochemical or physiological properties [13]. This classification can be sensitive to single mutational events (see Section 2.3). Biovars were described in many environmentally and clinically relevant genera such as *Rhizobium*, *Pseudomonas*, *Serratia*, *Ralstonia*, *Mycobacterium*, *Salmonella* and *Brucella*.

Rep-PCR enabled to differentiate genotype groups of strains belonging to *Pseudomonas fluorescens* bv. I. Strains of this biovar showed particular preference for the mycorrhiza and mycorrhizosphere of *Laccaria bicolor*, whereas strains of other biovars were more frequently isolated from soil [29]. Genotype clusters within biovars of *P. fluorescens* and *Pseudomonas putida* were distinguished using rep-PCR and correlated with the phenotype clusters obtained for the strains [30].

The use of the pathovar and biovar systems is highly inconsistent. It seems possible to revise the pathovar and biovar concepts, and base affiliation within a species primarily on genotype groups with defined genetic distances assessed by high resolution fingerprinting approaches. These clusters, for which description e.g. as subspecies (see Section 2.2) can be useful, may be subdivided by specific phenotypic traits such as host preferences or substrate utilization as pathovars or biovars.

Serovars are particularly important in medical disciplines and are distinguished by distinctive antigenic properties. They are described in the genera *Salmonella*, *Vibrio*, *Mycobacterium*, *Listeria*, *Leptospira*, *Chlamydia*, *Actinobacillus* and *Bacillus*, for example. As with the biovar system, serovar categorization can be highly sensitive to single mutations. Resolution of serotyping depends on the specificity of the antibodies, and these may exhibit unexpected cross-reactivity. The use of antibodies for taxonomic purposes must be evaluated judiciously because they do not necessarily track taxonomic entities. Serotyping can be extremely useful in epidemiological and virulence studies, particularly in combination with other sensitive methodologies (e.g. [31]). For example, Dogan et al. [32] showed that *Actinobacillus actinomycetomitans* serovar e comprises several biovars and genomovars. Serovar 1 and serovar 5 strains of *Actinobacillus pleuropneumoniae* were genotypically different [33]. However, Saunier et al. [34] found no correspondence between serovar groups and host specificity and only in certain cases close association between serovar and taxonomic groups of *P. syringae* pathovars. The authors hypothesized that the serological homogeneity among several pathovars may be attributed to an ancestor which presented the different O-antigenic epitopes and diversified later to the respective pathovars.

Phagovars are important in epidemiological analyses. They are distinguished by their ability to be lysed by certain bacteriophages. Phagovars are particularly used in clinically relevant taxa such as *Yersinia*, *Salmonella*, *Staphylococcus*, *Listeria monocytogenes*, *Corynebacterium diphtheriae*, *Pseudomonas aeruginosa* or *Escherichia coli*.

The following categorization terms used for infrasubspecific ranks [13] do not have widespread use. Forma specialis is ancient and pertains to a parasitic, symbiotic or commensal organism distinguished primarily by adaptation to a particular host or habitat. Formae speciales are found e.g. in *Rhodobacter sphaeroides*, *Chlorobium limicola* and *Staphylococcus aureus*. Cultivar (e.g. in *C. diphtheriae*) pertains to special cultivation properties, chemovar (e.g. in *Vibrio alginolyticus*) to production of a particular chemical, chemoform to chemical constitution, and morphovar to special morphological characteristics.

2.5. Methods of resolving microdiversity

Problems to achieve consensus in polyphasic taxonomy can arise e.g. from the comparatively low resolution of *rrs* comparison, the high resolution of phenotyping methods and/or the lack of reproducibility of some biochemical characters [4,5,35]. A list of abbreviations of techniques used in molecular biotyping is shown in Table 1.

A variety of genotyping methods such as rep-, AP- and inter-LINE-PCR, random amplification of polymorphic DNA (RAPD) or AFLP and SCAR analyses, restriction fragment length polymorphism (RFLP) analysis of total or partially amplified genomic DNA, and analyses of the 16S/23S rDNA spacer region are meanwhile available which provide the required high resolution between strain and species levels. Information on these techniques is found in [4,36–41]. These methods provide high taxonomic resolution and can serve as a fast molecular chronometer by which initial genome diversification and evolutionary speciation may be detected. They are easily performed and standardized, and have been successfully applied in a variety of medical, agricultural, industrial and environmental studies on microbial diversity, identification and classification ([4,42] and references therein).

For the assessment of genetic diversity, working with clone libraries is labor intensive. Techniques such as denaturing or temperature gel gradient electrophoresis (DGGE, TGGE) allow separation of distinct PCR amplicons from environmental DNA without prior cultivation [43]. Taxon-specific primers, which amplify regions providing higher resolution than the *rrs* gene, have been developed for *Ochrobactrum* species (M. Lebuhn et al., unpublished), and the diversity of DGGE/TGGE-separated and sequenced amplicons is being analyzed. Our knowledge of microbial diversity will profit from such fine-tuning approaches.

Comparative studies within different genera using such genotyping methods simultaneously with phenotyping, DNA/DNA hybridization and *rrs* homology approaches (e.g. [17,44,45]) prove that they are particularly useful for studies on intraspecific microbial diversity. Rep-PCR, AFLP and ribosomal spacer analyses were efficient in determining genetic distances delineating genomospecies of *P. syringae*, and they can serve as an estimate of DNA relatedness [17,27]. A high correlation between rep-PCR, AFLP-generated genotype and DNA hybridization groups was obtained for a large number of *Xanthomonas* and *S. maltophilia* strains, suggesting that the genomic finger-

Table 1
Abbreviations and acronyms for biomolecular techniques

Abbreviation	Standing for	Reference or review
AFLP	**a**mplified (DNA) **f**ragment **l**ength **p**olymorphism	[4,41]
AP-PCR	**a**rbitrary **p**rimed **p**olymerase **c**hain **r**eaction (of DNA)	[41]
ARDRA	**a**mplified **r**ibosomal **D**NA **r**estriction **a**nalysis	[41]
BOX-PCR	**p**olymerase **c**hain **r**eaction of (DNA sequences between) **BOX** elements	[40,42]
DGGE	**d**enaturing **g**el **g**radient **e**lectrophoresis	[43]
ERIC-PCR	**p**olymerase **c**hain **r**eaction of (DNA sequences between) **e**nterobacterial **r**epetitive **i**ntergenic **c**onsensus sequences	[40,42]
FAME	**f**atty **a**cid **m**ethyl **e**ster **a**nalysis	[4]
IGS (analysis)	**i**nter**g**enic **s**pacer analysis (= ITS analysis = RISA when used for ribosomal spacers)	[38]
Inter-LINE-PCR	**p**olymerase **c**hain **r**eaction of (DNA sequences between) **l**ong **in**terspersed **e**lements	[39]
ITS (analysis)	**i**nternal **t**ranscribed **s**pacer analysis (= RISA)	[38]
MLEE	**m**ulti**l**ocus **e**nzyme **e**lectrophoresis	[36]
PFGE	**p**ulsed **f**ield **g**el **e**lectrophoresis	[4,41]
PCR-RFLP	**r**estriction **f**ragment **l**ength **p**olymorphism of **p**olymerase **c**hain **r**eaction-generated amplicons	[4,41]
RAPD-PCR	**p**olymerase **c**hain **r**eaction of **r**andom-**a**mplified **p**olymorphic **D**NA	[41]
RC-PFGE	**p**ulsed **f**ield **g**el **e**lectrophoresis (of DNA digested by) **r**are **c**utting endonucleases	[41]
REP-PCR	**p**olymerase **c**hain **r**eaction of (DNA sequences between) **r**epetitive **e**xtragenic **p**alindromic elements	[40,42]
rep-PCR	**p**olymerase **c**hain **r**eaction of (DNA sequences between) **rep**etitive (REP, ERIC or BOX) elements	[40,42]
RISA	**r**ibosomal **i**ntergenic **s**pacer **a**nalysis	[38]
SCAR	**s**equence-**c**haracterized **a**mplified (DNA) **r**egions	[37]
TGGE	**t**emperature **g**radient **g**el **e**lectrophoresis	[43]

printing methods can be used to determine the taxonomic diversity and phylogenetic structure of bacterial populations [28]. They can be particularly useful in combination with less sensitive *rrs* homology analyses to reconstruct evolution of lineages at different scales of resolution. Since standardized genomic approaches have a higher resolution power than DNA/DNA hybridization techniques, they are more suitable for the determination of microdiversity. However, DNA/DNA reassociation data are strictly necessary to define the species before dissecting by genomic fingerprinting. Therefore integration of genomic fingerprinting approaches into polyphasic taxonomy can lead to a better understanding of speciation processes and identification of evolutionary driving forces, and thereby may help to improve definitions of species and lower taxonomic ranks.

The high resolution phenotype fingerprinting method, multilocus enzyme electrophoresis (MLEE), is appropriate to determine the extent of genetic recombination between members of a population. This type of analysis can support results of high resolution genetic fingerprinting in determining the clonality or the degree of sexuality within a population [46].

3. Molecular mechanisms generating microdiversity in bacteria

Genetic variation is a prerequisite for biological evolution. The basic genetic sources and non-genetic factors contributing to the generation of mutants have recently been reviewed [47]. These include point mutations, which lead to a stepwise improvement of available biological functions on which natural selection exerts evolutionary pressure. It was hypothesized that mutation frequency may be increased dramatically under some environmental stress situations, giving rise to episodic rapid evolution or macroevolution [48]. Chromosomal rearrangements in bacterial species can give rise to diversification and may lead to phenotypes with different abilities to occupy ecological niches [49,50]. Gene conversions or duplications are products of intragenomic recombination. Random recombination of gene loci and net-like evolution may lead to the development of separated lineages [51,52]. Finally, DNA acquisition results in new capacities in a single step which could be very advantageous for the colonization of new habitats. Horizontal gene transfer is widely recognized as the mechanism responsible for the distribution of antibiotic resistance genes, gene clusters encoding biodegradative pathways and pathogenicity/symbiosis determinants [52]. Bacterial conjugation and transduction as well as transformation [53] are well known natural tools of genetic exchange in bacteria. Events of horizontal gene transfer could initiate further evolutionary processes and may thus be key steps in creating microdiversity up to the level of speciation. For example, non-toxigenic *Bacteroides fragilis* strains seem to have evolved by horizontal gene transfer from very different organisms [54].

It was hypothesized that the appearance of different types of spontaneous mutations is influenced by 'evolution genes' which could act as generators or modulators of the frequency of genetic variation [47]. New evidence for the existence of different sets of evolutionary mechanisms arose from the information of the first complete genomic sequences of different strains of *Helicobacter pylori, Escherichia coil, Salmonella typhimurium* and *Neisseria meningitidis*. Extensive studies using molecular typing techniques had demonstrated that gene content and rearrangement are highly variable between different *H. pylori* strains [55]. The availability of two *H. pylori* genome sequences permitted direct comparison of gene sequences providing detailed information on the particular mutations which occur [56]. The genomic map of the *H. pylori* strains J99 and 26695, constructed using the *Not*I restriction enzyme and PFGE, was consistent in size with that predicted by the genome sequence [57]. The size differences of the restriction fragments between the two strains are mainly the result of synonymous mutations, which are mutations at the third position of the codons and therefore not translated into protein sequence changes. The level of this synonymous divergence is comparable between the human pathogens *H. pylori* and *N. meningitidis* (K_s = 15–21%) but much lower in *E. coli* (K_s = 6.6%). Therefore, the results obtained using lower resolution typing techniques may result in an overestimation of the extent of genetic diversity as compared to the real phenotypic diversity. The frequency of non-synonymous mutations is also higher in *H. pylori* than in *E. coli* (K_a = 2.5 vs. 0.2%) [58]. In addition, significant differences in the gene arrangement between the two strains were apparent. Inversion and/or transposition of 10 homologous regions are required for an alignment of the two chromosomes. Microdiversity of individual gene sequences occurs at different frequencies in *H. pylori* strains, as compared to *S. typhimurium* and *S. typhi* [58]. In addition, the sequence comparison of six genes (*putP, pabB, sppA, zwf, trpB* and *trpC*) in *E. coli* with their counterparts in *H. pylori* revealed a much higher variation (by pairwise difference) within *H. pylori* both at the nucleotide and amino acid level [56]. This agrees with the observed allelic diversity analyzed by MLEE in *H. pylori*, which is higher than in any other bacteria yet examined. In addition, pathoadaptive mutations could be involved during a single infection contributing to the population structure of a pathogen. During a *H. pylori* infection of gnotobiotic piglets, Akopyants et al. [59] demonstrated that the introduced strain initially grew weakly but became more adapted during several serial passages.

The whole genome sequences of *H. pylori* were compared to identify potential mutator genes [56]. Several candidate mutator genes were found, but most of these genes have only a low or intermediate level of identity to their equivalents of known function in other bacteria, and

therefore, their true functions are uncertain. It is known that mutation repair activity is not constant, but can be considerably increased in response to environmental and physiological signals [60]. The *E. coli* SOS response is the best known case of a transient mutator response. The SOS response includes different repair functions, which simultaneously lower replication fidelity and increase the appearance of mutations [61]. While a *recA* (SOS activator) homologue was found in *H. pylori*, a *lexA* gene (SOS repressor) and the sequence motifs for LexA binding sites are absent. The constitutive expression of the SOS system or other unidentified regulatory mechanisms in *H. pylori* may contribute to the higher mutation frequency. In addition, genes encoding UmuD, UmuC and DinB proteins, three major components of *E. coli* SOS mutagenesis, were not found in the *H. pylori* genome sequences.

On the basis of different apparent mutation frequencies in different bacterial species, one can speculate that bacteria originating from more or less stressful habitats contain different sets of potential mutator genes. For example, replication fidelity is maintained at very high levels by a number of error-avoidance mechanisms, that operate before, during or after replication. Mutations in the *mutHLS* system are known to increase point mutations up to 1000-fold [62]. In contrast to *E. coli*, *H. pylori* does not contain *mutH* and *mutL* genes, which encode important components of purin/pyrimidine mismatch repair system in *E. coli*. This could explain the observation that transition mutations predominate, when the two existing genomes of *H. pylori* are compared [56]. Le Clerc et al. [63] observed high mutation frequencies among *E. coli* pathogens that were attributed to a deficiency in the *mutHLS* high fidelity DNA repair system.

In summary, there is evidence from genomic sequence comparisons, that recombination and mutation events do contribute considerably to genetic flexibility, and are major parts of the genetic adaptation to changing environmental conditions. These mutation frequencies are different in certain bacterial groups and are apparently based on a different genetic background of mutator or evolution genes. However, more experimental evidence is needed to confirm these attractive ideas.

4. Examples for bacterial microdiversity

4.1. Factors influencing microdiversity in soil and rhizosphere bacteria

4.1.1. Burkholderia

An interesting model species for studies on environmental influences on microdiversity of bacteria is *B. cepacia*, because a great variety of strains with different phenotypic features like plant growth promotion [64], biocontrol of soilborne phytopathogen [65], plant pathogenicity [66], human pathogenicity [67] and pollutant degradation [68] is known.

The utility of 16S–23S rDNA spacer (ITS) analysis in molecular epidemiology of *B. cepacia* was shown by Kostman et al. [69]. RFLP patterns were identical for clinical isolates implicated in person to person transmission, and different from unrelated isolates.

The microdiversity of a *B. cepacia* population from the rhizosphere of maize was studied by di Cello et al. [70] using molecular typing methods in order to assess the degree of root association and microbial diversity at five stages of plant development. The affiliation of the strains to *B. cepacia* was confirmed by rrs-ARDRA patterns and sequence analysis. Microdiversity was assessed using the RAPD technique and analyzed using the molecular variance (ANOVA) method. Throughout the study, *B. cepacia* was strictly associated with maize roots and presented 0.6–3.6% of the total culturable rhizosphere microflora. Microdiversity among the 83 isolates was high and was dependent on the sampling time. Microbial genetic variability was markedly higher in the first stages of maize root growth.

Dalmastri et al. [71] compared the effects of soil type, maize cultivar and root location on the microdiversity of root-associated *B. cepacia* populations using 180 bacterial isolates. The results indicated that among the factors studied, the soil was clearly dominant in affecting the genetic diversity of maize root-associated *B. cepacia* populations. The percentage of variation among populations was significantly higher between *B. cepacia* populations recovered from maize planted in different soils than between *B. cepacia* populations isolated from different maize cultivars and from distinct root compartments such as rhizosphere and rhizoplane. Analysis of the genetic relationship among *B. cepacia* isolates revealed the presence of bacterial populations with frequent recombination and a non-clonal genetic structure. Strains showed clustering on the basis of their origin, which confirmed the major effect of soil type on the genetic diversity of these *B. cepacia* populations. In contrast, strains from the rhizosphere and rhizoplane did not differ significantly in their genetic structure. Neither study provided evidence of how the genetic variability affects the phenotype.

Yohalem and Lorbeer [66] studied the intraspecific metabolic diversity among 218 strains of *B. cepacia* isolated from onions, soils and clinical specimens. Each strain was characterized on the basis of selected phenotypic markers such as virulence to onion or catabolic ability using the Biolog-GN® plate approach. Several statistical methods (Gower similarity, pattern difference and Jaccard similarity) were used to calculate similarity. As in the work of Dalmastri et al. [71], a strong influence of soil type was found. Strains isolated from the same field were frequently found to be phenotypically similar. However, strains isolated from different sites with similar cropping history

were not. Strains isolated directly from onion slices formed a significant cluster and were all virulent to onion.

4.1.2. Ochrobactrum

Ochrobactrum is a ubiquitous soil bacterium, and can be a human pathogen [72]. Some *Ochrobactrum* isolates belonging mainly to the species *O. anthropi* are known as degraders of different pollutants [73]. The environmental distribution and microdiversity of *Ochrobactrum* spp. was studied by Schloter et al. [74], comparing a fallow land soil and a soil with maize cultivation. The isolates were obtained by immunotrapping with a specific monoclonal antibody [74]. Four hundred isolates were characterized genotypically by rep-PCR profiling. Among the isolates from the fallow land, seven different genotypes were identified which fell into two distinct clusters. The distribution of the isolates from this site was very constant over a year's period, with two dominant genotypes at each sampling time. Although the richness of genotypes was similar in the soil cultivated with maize (eight different genotypes), these genotypes were different from those obtained from the fallow land. The frequency of change in occurrence of the different genotypes was much higher and could be correlated with different plant developing stages.

Schloter et al. [75] investigated the influence of different farming practices on the microdiversity of *Ochrobactrum* spp. Plots under conventional or 'ecological' farming for 2, 8 and 40 years, respectively, were investigated. In the year of sampling, winter wheat was cultivated on all plots. After immunotrapping and classifying the strains by the ECO-Biolog® system, 24 phenotypes could be distinguished. No significant differences in phenotype variability were observed in plots with conventional farming and the plots which had been under ecological farming for 2 or 8 years. In contrast, in the soils which had been ecologically farmed for 40 years, marked changes in the presence of pheno- and genotypes were found.

In a recent study [45], 186 *Ochrobactrum* strains were immunotrapped from soil and wheat root tissue, and two new species (*O. grignonense* and *O. tritici*) were described. For the comparison of structural and functional (intra)-species diversity in both habitats, rep-PCR and substrate utilization analyses (Biolog-GN® and two API® systems) were performed. One rep-group of *O. grignonense*, one of *O. intermedium* and four *O. anthropi* rep-groups were isolated only from soil, and one rep-group of *O. tritici* only from root tissue. Strains of two *O. anthropi* rep-groups were found in both compartments.

Concomitant with the higher intraspecies diversity of *O. anthropi* (and the higher intrageneric diversity of *Ochrobactrum*) in soil vs. wheat root tissue, *O. anthropi* strains from soil exhibited higher substrate utilization versatility and capacity (Lebuhn, unpublished). Plant root conditions seem to select for distinct genotypes that are adapted to and competitive in the rhizosphere environment. These genotypes may have lost the abilities of distinct soil ancestors to use certain substrates. The very low genotypic diversity of *O. tritici* strains and their high DNA similarity to *O. anthropi* [45] indicates that they may have diverged recently from ancestor strain(s) that were similar to *O. anthropi*. Upon colonization of the niche plant rhizoplane, *O. tritici* strains may multiply particularly quickly and clonally.

Similarly, distinct genotypes of *O. anthropi* and *O. intermedium* became virulent as outbreak strains that were isolated from human patients [76,77].

4.1.3. Stenotrophomonas

S. maltophilia is, like *O. anthropi*, an interesting species to study factors giving rise to intraspecies diversity. It is a common and ubiquitous species, increasingly important as an opportunistic human pathogen, and may be involved in degradation of xenobiotics.

Hauben et al. [78] analyzed genotypic intraspecies diversity of 108 *S. maltophilia* strains from different clinical and environmental sources using AFLP genotyping analysis, DNA/DNA hybridization and sequence comparison of *rrs* genes. They found no significant phenotypic differences between the strains, but they showed a high genotypic heterogeneity. Three genomovar/genospecies clusters containing (almost) exclusively clinical isolates, three clusters with (almost) exclusively environmental isolates and four mixed clusters were delineated. Distribution of the strains to the clusters and genotypic diversity within the clusters were similar. AFLP analysis provided the highest and *rrs* sequence comparison the lowest resolution. AFLP pattern similarity of 45% corresponded to 70% DNA/DNA reassociation and 98.5% *rrs* sequence identity. AFLP analysis even allowed distinction between colony variants of certain *S. maltophilia* strains (see also Section 2.3).

4.1.4. Pseudomonas

The most interesting background of diversity studies on *P. fluorescens* is that strains of this species can cause stimulation and damage to plants. However, most of these studies suffer from the incomplete taxonomy of the species related to *P. fluorescens* ('fluorescent pseudomonads') and/or the lack of sound underlying taxonomic data at the subspecies level. For example, when the genetic structure of fluorescent *Pseudomonas* was studied, an overall clonality and an 'ecotypic' structure of the populations were demonstrated, but there was evidence for parasethal recombination among specific groups of these populations [79], raising questions about their correct taxonomic position. In the context of a diversity analysis of fluorescent pseudomonads in soils and in plant rhizosphere, it was shown that genotypic microdiversity was higher in soil than in plant tissue, and that the genotypic and phenotypic diversity was predominantly influenced by the plant host (tomato vs. flax) [30]. The two host plants differentially selected for the presence of certain biovars corresponding to distinct REP-clusters in root tissue. *P. putida*

bv. A was genotypically and *P. fluorescens* geno- as well as phenotypically heterogeneous, and *P. fluorescens* bv. II was more closely related to *P. putida* than to the other *P. fluorescens* biovars. These data suggest, in agreement with earlier studies, that *P. fluorescens* should be taxonomically revised.

As in many other studies on Gram-negative bacteria, Latour et al. [80] observed that the diversity of fluorescent pseudomonads was higher in soil than in plant tissue. The plant host and, particularly, the soil type influenced diversity, but no clear effect on biovar distribution was observed. A low degree of genetic diversity within *P. fluorescens* on the rhizoplane of corn was obtained by MLEE analysis [81]. There was again no clear evidence that all strains studied for microdiversity belonged to the same species (*P. fluorescens*). Using phenotypic (Biolog®) and genotypic (rep-PCR) markers, Frey et al. [29] also obtained higher intraspecies diversity of *P. fluorescens* in soil, compared to the diversity of *L. bicolor* on the mycorrhizoplane.

The only work dealing with the diversity of a non-fluorescent *Pseudomonas* species below the species level was carried out with *Pseudomonas corrugata*. The analysis of genotypic diversity (rep-PCR) of *P. corrugata* strains isolated from two contiguous field plots under continuous wheat cropping or wheat-maize crop rotation revealed that most of the genotypes were present in both plots. Comparison of the richness and evenness diversity indices (global diversity) indicated no significant difference between the plots, whereas rep-PCR fingerprinting data (intraspecific diversity; 291 strains assigned to 55 ERIC-PCR profiles) showed that the genetic structure of *P. corrugata* was significantly affected by the crop management [82]. The strains were assigned to *P. corrugata* using PCR-RFLP of the *rrs* gene.

Molecular fingerprinting procedures including RAPD, rep-PCR with REP, ERIC and BOX primers, and MLEE were used for the genotypic characterization of *P. stutzeri* isolates from marine and waste water, and from clinical and soil samples. High genotypic diversity was found within *P. stutzeri*. Combined cluster analysis (UPGMA) of RAPD-, rep-PCR and MLEE fingerprints was superior to the cluster analysis of individual typing approaches, and differentiated *P. stutzeri* into seven distinct genotypic groups. The subdivision of the species into several genomovars was supported and the grouping of the isolates according to *rrs* gene phylogeny was reproduced at higher resolution [46].

4.1.5. Paenibacillus

Paenibacillus polymyxa is one of the most common Gram-positive inhabitants of soil and the plant rhizosphere. In contrast to results from many studies on Gram-negative bacteria showing that their population size is increased up to 1000-fold in the rhizosphere, the numbers of *P. polymyxa* strains were similar in soil and in the rhizosphere [83]. This controversy may, in part, be due to different definitions of the spatial extension of 'the rhizosphere' effect. Studying the structural and functional bacterial diversity in a single wheat field site harboring a common pool of bacterial diversity, a decreased genotypic (RFLP of *rrs* gene) and phenotypic (API® system) diversity from soil to rhizoplane was observed [83]. Clonal growth of some *P. polymyxa* strains on the root system could explain this decreased diversity on the rhizoplane.

In a recent work reporting on genotypic (rep-PCR) and phenotypic (API® system) diversity of *P. polymyxa* in Algerian soils, a highly significant effect of the soil type was found [84]. The genetic structure of the populations was completely different from one soil to the other. None of the various *rep*-PCR profiles was found to be common in the different soils. A decreased diversity in the wheat rhizosphere after repeated wheat cultivation was also noticed.

The analysis of the genotypic (rep-PCR) diversity of the related root-associated species *Paenibacillus azotofixans* populations isolated in Brazilian clay soils evidenced a significant effect of the soil type [85]. A high phenotypic and genotypic (RAPD, rep-PCR) diversity within *P. azotofixans* populations isolated from the rhizosphere of wheat, sugar cane, sorghum and maize was found in a study with Brazilian soils [86]. Due to the high microdiversity, it was impossible to obtain clear genotypic clusters relative to plant origin.

4.2. Factors influencing microdiversity in symbiotic and plant pathogenic bacteria

4.2.1. Bradyrhizobium, Sinorhizobium and Rhizobium

Rhizobia are interesting bacteria to study the influence of the plant host on microdiversity, since mostly a symbiotic interaction between plant and microbe is formed, but the taxonomy of rhizobia is highly complex and matter of debate.

In a study of Laguerre et al. [87], rhizobial diversity at the genus and species level was independent of the geographic origin and the host plant affinity. Zhang et al. [88] investigated the phylogeny and diversity of 22 *Bradyrhizobium japonicum* strains isolated from the root nodules of two peanut cultivars from four different sites in China using phenotypic markers (growth rate, fatty acid methyl ester analysis (FAME) and a genotypic marker (rep-PCR)). The fingerprints clearly indicated a strong influence of the plant cultivar. In this study on symbiotic bacteria, a grouping of the isolates according their geographic origin was possible. Urtz and Elkan [89] also investigated the influence of peanut plants on the diversity of 33 *B. japonicum* populations. In contrast to Zhang et al. [88], they did not fingerprint the whole genome for classification of the isolates, but only the symbiotic *nif* and *nod* genes. It could be shown that for most isolates these two markers were very stable and showed low genetic variability.

Genetic diversity of 96 *Sinorhizobium meliloti* populations in nodules of different *Medicago sativa* varieties was studied by Paffetti et al. [90,91]. Although all isolates were phenotypically indistinguishable, genotypic differences using RAPD-PCR and ANOVA were found. The results indicated that there was significant genetic difference among strains nodulating different varieties of *M. sativa*, which showed that the plant genotype is a major factor in shaping the genetic structure of *S. meliloti*.

Souza et al. [92] investigated the genetic structure of *Rhizobium leguminosarum* bv. phaseoli populations associated with wild and cultivated beans over several spatial scales, ranging from individual host plants to throughout the western hemisphere. Genetic differences were found at all scales investigated, even as small as a cultivated plot. However, the amount of genetic variability was much greater among isolates collected throughout the western hemisphere than among isolates from one site. The authors concluded from their results that the limited migration between populations contributes substantially to genetic variability in *R. leguminosarum*.

An interesting example of horizontal gene transfer was reported for four genomovars of non-symbiotic *Rhizobium loti* strains, which had acquired a chromosomal 'symbiotic island' from an inoculant strain [93]. The symbiotic and non-symbiotic strains were almost isogenic.

Detailed genotyping results suggest that *R. leguminosarum* and its biovars require taxonomic revision. Data on DNA relatedness show that *R. leguminosarum* bvs. trifolii, viciae and phaseoli represent three different genomospecies, and that a new hitherto undescribed *Rhizobium* species is present [94]. Rep-PCR and PCR-RFLP of the 16S–23S rDNA ribosomal spacer produced similar clusters at two levels, for *R. leguminosarum* bv. viciae strains as well as for a collection of *R. leguminosarum* bvs. trifolii, viciae and phaseoli strains. This grouping disagreed with the clusters obtained using PCR-RFLP of *nod* and *nif* genes which were in accordance with the biovar classification [95,96]. Since *nod* and *nif* genes are found on the Sym plasmid which is exchanged between soil microbial populations, and specific interactions may occur between chromosomal and plasmidic markers [97], it is highly probable that the biovar classification system of *R. leguminosarum* is based at least partially on features that are subject to gene transfer.

4.2.2. Frankia

Richie and Myrold [98] studied the geographical distribution and genetic diversity of *Frankia* strains infective in *Ceanothus* sp. Since until now no *Frankia* strain that can reinfect the host plant has been isolated from *Ceanothus* sp., DNA was isolated directly from the nodules, and the ribosomal spacer between 16S and 23S rDNA, including the flanking regions, was amplified. The PCR products were subjected to restriction enzyme digestion with 12 enzymes and RFLPs were compared. Using this technique, intraspecies diversity in *Frankia* populations from different nodules could be found. Surprisingly, the groups did not follow the taxonomic lines of the *Ceanothus* host species but showed a strong correlation to the geographical distribution of the sampling sites.

Clawson and Benson [99] studied the diversity of *Frankia* strains in root nodules from plants of the family *Myricaceae*. The authors found that one host plant could be colonized by different *Frankia* strains. The strain eveness varied greatly between the plant species. The results indicate that the influence of the host plant on the diversity of the symbiotic *Frankia* population may be low. This would be in contrast to the findings with rhizobia.

4.2.3. Pseudomonas and Xanthomonas

P. syringae and *Xanthomonas campestris* comprise major pathogens of various important crop plants. Taxonomy within these species is complicated and may be subject to revision.

A microdiversity study of *P. syringae* populations based on PCR-RFLP of internal transcribed spacer (ITS) [27] confirmed that this genetic fingerprinting method is more discriminatory than DNA/DNA hybridization, but less discriminatory than pathogenicity groups. There was a global assessment of these pathogenicity groups using PCR-RFLP of ITS. Additional data on *P. syringae* demonstrated the usefulness of RAPD and AFLP (there was a good correlation between both techniques) to provide evidence of evolutionary differences between *P. syringae* pv. tomato and *P. syringae* pv. maculicola, suggesting that the host plant could be an important factor in the plasticity of the bacterial genome [17].

Using rep-PCR fingerprinting, different genotypic patterns were obtained for strains of various *X. campestris* and *P. syringae* pathovars. Genotypic diversity in pathovars with a broad host range such as *P. syringae* pv. syringae and *X. campestris* pvs. campestris and vesicatoria was generally higher than in pathovars with a restricted host range [100]. *P. syringae* pv. syringae isolates which are adapted to a distinct niche were genotypically homogeneous and may be the result of recent adaptation and/or genetic isolation [101].

4.2.4. Ralstonia

R. solanacearum, the causal agent of bacterial wilt, is a complex taxonomic unit which is subdivided in races and biovars. It affects many important crop species and is found in various climates.

Smith et al. [102] investigated the microdiversity within *R. solanacearum*. ERIC- and BOX-PCR and PFGE were used for genotypic fingerprinting of 45 isolates from Kenya and 35 isolates from other countries. A biogeographic trend was clearly visible, showing that the isolates from Kenya form a distinct cluster, which could only be analyzed in more detail by PFGE. Further evidence for different evolution in *R. solanacearum* caused by spatial sepa-

ration was found by Poussier et al. [103]. PCR-RFLP analysis of the *hrp* gene region of a worldwide collection of *R. solanacearum* strains resulted in continent-specific grouping of biovars and of a strain group within biovar 1.

5. Conclusions

Many examples show that there is an important diversity of bacteria below the species level, and underline the need to prove identities by polyphasic taxonomy. High resolution genotype fingerprinting techniques are of central importance in microdiversity analysis. However, DNA/DNA reassociation methods are required to show that the bacteria under investigation belong to the same species. It is recommended that species are subdivided into subspecies, which may be delineated by the genetic distances obtained from genotype fingerprinting methods. Taxonomic revision is required for several genera.

Various influences on bacterial microdiversity have been identified, such as spatial separation, habitat differences and specific bacterium–host interactions. These influences can apparently differ between one species and another, and must be subject of specific investigation in each case. Microdiversity in symbiotic and pathogenic bacteria seems to be lower than in 'free living' bacteria. This may be due to the unidirectional and predominating influence of the host, whereas bacteria exposed to a variety of selective forces may have maintained a higher adaptative capacity. As there is enormous microdiversity in bacterial populations, reliable sampling strategies must be performed to improve data from in this field experiments.

Limited DNA repair and replication fidelities may not be considered as errors or accidents but rather are welcome tools to produce genetic microdiversity. Since these mutator gene systems are regulated or modulated themselves and evolved differently in bacteria of different ecology, they can be considered as active and major players in the generation of bacterial microdiversity. A rich biodiversity guarantees long-lasting maintenance of a bacterial population and development of new evolutionary lines.

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