

On the necessity to study natural bacterial populations - the model of *Bacillus simplex* from "Evolution Canyon" I and II, Israel.

Running Title: Evolutionary study of natural bacterial populations

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Abstract

How do bacteria evolve and speciate in natural environments? How does bacterial evolution relate to bacterial systematics? Exploring these answers are essential because bacteria profoundly impact life in general, particularly, humans. Much insight into bacterial microevolution has come from theoretical and computational studies and from multigenerational laboratory systems (“Experimental Evolution”). These studies, however, do not take into account the diversity of modes of how bacteria can evolve under the complexity of the real world, e.g., nature. We argue, therefore, that for a comprehensive understanding of bacterial microevolution, it is essential to study natural populations. We underline our argument by introducing the *Bacillus simplex* model from “Evolution Canyon”, Israel. This metapopulation splits into different evolutionary lineages that have adapted to the microclimatically different slopes of “Evolution Canyon”. It was shown that temperature stress is a major environmental factor driving the *B. simplex* adaptation and speciation progress. Therefore, this model population has proven highly suitable to study bacterial microevolution in natural habitats. Finally, we discuss the *B. simplex* intrapopulation divergence of lineages in light of current controversies on bacterial species concepts and taxon identification.

Keywords: natural bacterial populations, theory-based modern taxonomy, *Bacillus simplex*, “Evolution Canyon“, environmental stress, Stable Ecotype Model

Bacteria: The unseen majority of biological diversity

The prokaryotic microbial world (the domains of Bacteria and Archaea) represents the unseen majority of biological diversity of this planet (Whitman et al., 1998; Torsvik et al., 2002; Gans et al., 2005). Prokaryotic microbes (hereafter simply called bacteria) reside in nearly every habitat and can even survive extraterrestrial environments (Nicholson et al., 2000). The total amount of prokaryotic carbon is similar to the total carbon in plants, however, the total amounts of nitrogen or phosphorus fixed in prokaryotes approximately exceed the amounts fixed in plants by 10-fold (Whitman et al., 1998). The number of bacterial species probably exceeds tens of millions of species (Gans et al., 2005). A huge bacterial diversity has been identified in environmental communities (Amann, 2000; Pernthaler and Amann, 2005). Life of all higher organisms is ultimately dependent on bacteria. The important catabolic and anabolic biological transformations of organic material, indispensably necessary for the eukaryotic food chain, are performed by bacteria. Also, bacteria can impact our lives by their pathogenicity. For example, *Pseudomonas aeruginosa* and *Burkholderia cepacia*, which are both abundant in natural habitats, such as soil and sediments, are severe human pathogens frequently causing high mortality rates in, e.g., bacteremia and cystitis fibrosis (Aris et al., 2001; De Soyza et al., 2001; Osmon et al., 2004; Zavascki et al., 2006).

The ultimate importance of bacteria for life in general, and, from a clinical perspective, specifically for humans, makes it essential to develop theoretical and pragmatic frameworks of studying their evolution. Moreover, by reconstructing the evolutionary history of bacteria in natural environments

fundamental yet unresolved questions in evolution can be addressed. Major questions concern both ecological and organismic factors. Among the bulk of environmental factors, which are the important ones that drive adaptation and speciation and which are of less importance or have no relevance at all? Also, by which genetic and physiological means do evolutionary genomic lineages respond to environmental stress? How and with whom do organisms interact or antagonize? Which types of molecular solutions are “chosen” by bacteria in order to realize the necessary phenotypes under given environmental stresses? If we want to better understand the microbial world in which we benefit from but yet are also threatened by, then the evolution of natural populations must be reconstructed and analyzed.

Strategies to approach bacterial microevolution

There are three strategic approaches of exploring bacterial microevolution. One way is by using theoretical and mathematical studies, which frequently include computational simulations. A second way is to perform laboratory evolutionary studies with living bacteria (“Experimental Evolution”). Finally, a third way is to isolate the organisms from the natural habitat in which they most probably evolved and to reconstruct their evolutionary history.

The first two approaches differ fundamentally from the third way and in the aspects of bacterial evolution they can explore. Both theoretical/computational studies and Experimental Evolution enable scientists to follow real-time evolution. These studies usually start from a defined digital or biological organism and allow it to evolve under strictly defined and controlled

environmental conditions. During the real-time evolutionary process, intermediates and final descendants are stored for subsequent analysis, e.g., by competitive fitness experiments. Both approaches have yielded fascinating insights into evolutionary processes. For example, Lenski and colleagues allowed *E. coli* to evolve for more than 30,000 generations, thereby adapting to specific environmental conditions, i.e., either to different carbon sources or to different temperatures (Elena and Lenski, 2003). Rainey and Travisano found that a clone of *Pseudomonas fluorescens* diverged within just three days into different ecotypes adapted to different physical and chemical environments within a static microcosm (Rainey and Travisano, 1998). Moreover, based on the *Pseudomonas fluorescens* model, researchers measured the effect of different ecological scenarios on the evolution of heterogeneity or pleiotropic fitness tradeoffs (Jessup et al., 2004; Kassen et al., 2004; MacLean et al., 2004). Also, Maharjan and colleagues showed extremely rapid adaptive radiation (within 90 generations) of *E. coli* growing in a chemostat (Maharjan et al., 2006). Dekel and Alon used a combination of computer simulation and Experimental Evolution. They first predicted, using computational simulation, evolutionary dynamics in the fine-tuning of expression levels of the lac operon in *E. coli*, and subsequently verified the predictions by Experimental Evolution (Dekel and Alon, 2005). Adami and colleagues, frequently together with Lenski, used the Avida software platform in order to allow digital organisms to evolve (Adami, 2006a). This “Digital Evolution” project paved the way to studies on the evolution of genome complexity and robustness (Lenski et al., 1999; Wilke et al., 2001). A major finding was that irreducibly complex organismal functions

can indeed evolve by building on simpler functions (Lenski et al., 2003; Adami, 2006b). In several cases this occurred surprisingly by using deleterious mutations as stepping stones (Lenski et al., 2003). Other authors were focused on the evolution of mutators (Tanaka et al., 2003) or the existence conditions of plasmids (Bergstrom et al., 2000). Recently, the theoretical basis of the dynamics of adaptation has been reviewed (Orr, 2005a; Orr, 2005b).

Fundamentally different from the above, *in silico* or *in vitro* approaches, is the reconstruction of the evolutionary history of natural populations. These populations represent the bacterial evolutionary biology in its entire complexity as a result of the reciprocal interaction of bacteria with nature. Computational studies and Experimental Evolution need to reduce complexity and to limit the numbers of parameters in order to yield robust conclusions. Nature, in contrast, represents the comprehensive reality in which all organisms live, benefit from, and are also threatened. For an enhanced understanding of the biological world we live in, it is therefore necessary to study natural populations. Despite our inability to control the parameters of the complex evolution of natural populations (which is an experimental disadvantage compared to *in silico* or *in vitro* approaches), the results of this process in itself are analytically accessible and will yield major insights into bacterial microevolution and speciation.

Different approaches to bacterial systematics – pragmatic versus theory-based taxonomy

In bacterial systematics, molecular cutoffs are applied to demarcate species. Generally, a threshold value of >30% variation between genomes as determined by DNA-DNA hybridization (and > 2 to 3% variation in 16S rRNA sequence) has been proposed as genetic distances needed to ensure that two strains belong to different species (Stackebrandt and Goebel, 1994; Rosselló-Mora and Amann, 2001; Cohan, 2002; Stackebrandt et al., 2002; Gevers et al., 2005). However, this pragmatic and widely accepted definition for the identification of the species taxon lacks reference to the biology of prokaryotes; i.e., a theoretical basis taking into account evolutionary forces that either cohere or drive apart biologically meaningful species (Cohan, 2002; Gevers et al., 2005). Interestingly, current bacterial systematics (Rosselló-Mora and Amann, 2001; Stackebrandt et al., 2002) frequently lumps together ecologically different populations in a single species (Palys et al., 1997; Cohan, 2002; Gevers et al., 2005).

According to current bacterial systematics, a species is a “category” (Rosselló-Mora and Amann, 2001; Stackebrandt et al., 2002; Coenye et al., 2005), which rather reflects the traditional Darwinian view, in which the species category is one of the ranks in the hierarchy of taxonomic categories such as varieties, genera, families, etc (Bock, 2004; de Queiroz, 2005a). The ranks differ from each other by the degree of differences within the rank members themselves (Darwin, 1859; de Queiroz, 2005a). During the Modern Evolutionary Synthesis, however, a shift in the conceptualization of species occurred. Species are now

viewed as fundamental categories of biological organization, similar to cells or organisms (Simpson, 1961; Mayr, 1969, 1982; de Queiroz, 2005a). Here, a species is a separately evolving metapopulation lineage (a single line of ancestry and descent) (de Queiroz, 2005a). The borders (and contingent properties) that cohere such metapopulation lineages are surely different between biological groups. Some may require intrinsic reproductive isolation, while others require shared specific mate recognition, phenetic differences, ecological distinctiveness, or monophyly, just to mention a few (de Queiroz, 2005b). For species that reproduce sexually, sexual isolation is the cohesive force that holds together a species and separates species from one another. But what are the factors that cause bacterial species to cohere? Recently, it was suggested that in predominantly asexual organisms, such as bacteria, compared to most eukaryotes, natural selection can be the force that determines the species (van Valen, 1976; Templeton, 1989; Cohan, 2002). This idea is implemented by the “Stable Ecotype Model” (Gevers et al., 2005; Cohan, 2006). The Stable Ecotype Model reflects, in principle, the metapopulation lineage concept: ecotypes have a history of coexistence (identified by sequence analysis), as well as a prognosis for future coexistence based on ecological distinctiveness (Gevers et al., 2005). Within the endeavors of establishing a theory-based prokaryotic taxonomy, a variety of models of bacterial evolution have been developed that take into account the differential impacts that selection, recombination, migration, and drift may have on different groups of bacterial organisms (Gevers et al., 2005; Cohan, 2006).

Here, we focus on evolution according to the “Stable Ecotype Model” (Gevers et al., 2005), since natural selection plays a profound role in the cohesion of bacterial species (Templeton, 1989; Cohan, 2002). Ecotypes are the ecologically distinct groups defined by genome-wide purging of diversity known as periodic selection (Cohan, 1994; Gevers et al., 2005). Periodic selection events occur when a new adaptive mutant arises within an ecotype, and natural selection causes the mutant and its nearly clonal descendants to replace all competing variants. Due to ecological differences between ecotypes, a periodic selection event within one ecotype does not extinguish the diversity within other ecotypes (Cohan, 1994; Majewski and Cohan, 1999). Thus, ecotypes have a history of coexistence (identified by sequence analysis, see contribution of Frederick M. Cohan in this special issue), as well as a prognosis for future coexistence based on ecological distinctness. Although genes can be exchanged between distantly related organisms (even across domains), the frequency of recombination (horizontal gene transfer), in probably most bacterial species, is too low to mix gene pools and thus keep ecotype populations from diverging (Cohan, 1994). Under the Stable Ecotype Model, new ecotypes are formed only rarely, and each ecotype endures many periodic selection events during its lifetime. Under these circumstances, there is time for an accretion of sequence divergence between ecotypes, with recurrent purging of diversity within but not between ecotypes (Gevers et al., 2005). Since bacteria are the most diverse group of organisms on earth, other models of bacterial evolution might also apply. These include models such as “Geotype”, “Geotype-plus-Boeing”, “Species-Less”, “Cohesive-Recombination”, “Recurrent

Niche Invasion”, and “Nano-Niche” (Gevers et al., 2005; Cohan, 2006). For example, hot-spring cyanobacteria seem to evolve according to the Geotype-model (Papke et al., 2003).

Summarizingly, it can be said that current bacterial systematics applies a very pragmatic and well accepted set of definitions in order to identify the species taxon as a taxonomic unit – but these definitions fail to identify of the central evolutionary units of bacterial diversity and ecology. This is nicely exemplified by the *B. simplex* model population we introduce here. We will show that the nomenclature *B. simplex* consists of several separately evolving ecological units.

What are the goals and procedures in reconstructing the evolutionary history of natural populations?

Only the contemporary generation is accessible for analysis. Thus, all conclusions must be derived from a retrospective or coalescent perspective. However, this is far more than simply producing a phylogenetic tree. The aim of such a study must be to obtain a coherent picture of the evolution of the investigated population by correlating DNA sequence based phylogeny (i.e., the genotype) with phenotype and ecology. Thus, special care must be taken to ensure that as much information as possible is available from the ecology of the sampling place. Frankly speaking, an isolate having no sampling record [when and where (geography and ecology) sampled] is worthless. As a first step, the phylogenetic history of the studied contemporary intraspecies organisms must be reconstructed with as much precision as possible. Phylogeny reconstruction

is the central element of evolutionary genetics. Suitable data may be obtained by, e.g., Multilocus Sequence Typing (Maiden et al., 1998). Second, it is also necessary to add information on the habitat of each individual in the phylogenetic tree from where it was isolated. This procedure may then allow the identification of groups of strains, members of which show genetic similarity (thus, being genetically cohesive) and occur in similar habitats (thus, belonging to the same ecotype) (a highly robust and objective method for identification of ecotypes, the “community phylogeny” method, is described elsewhere (Cohan, 2006) and in the contribution of Frederick M. Cohan in this special issue). Identification of such a strain-habitat distribution would allow the hypothesis that specific groups of strains have adapted to specific habitats. Finally, in order to support this hypothesis, it is necessary to perform as many ecological-physiological studies as possible. Identification of physiological traits that match the type of habitat would then confirm that the observed pattern of genetic distributions among habitats is not random. Having compiled an ideally large set of data during this procedure, the next step should reconstruct the evolution of the studied organisms during their adaptational divergence.

In the next sections we will present the natural bacterial model system of adaptive divergence that we are currently exploring.

The “Evolution Canyon” Model

The environmental evolutionary system of "Evolution Canyon" (“EC”) in Israel (Nevo, 1995, 1997, 2001) is highly suitable for studying the intertwined evolutionary processes of adaptation and speciation under microscale

environmental stress (Fig. 1). "EC" I (Lower Nahal Oren, Mount Carmel) and "EC" II (Lower Nahal Keziv, western Upper Galilee) are separated by 40 km and represent similar ecologically replicating canyons with sharply contrasting interslopes in each, separated by only 50-400 m (Nevo, 1995, 1997, 2001). The open park forest of warm-xeric, tropical, "African-Asian", savanna-like, south-facing "African" slopes (ASs) receives up to eightfold more solar radiation (Pavlicek et al., 2003) than the north-facing "European" slopes (ESs). Consequently, the ASs are warmer, drier, and spatiotemporally more heterogeneous and fluctuating and thus more stressful, than the green, lush, temperate, cool-mesic, "European" north-facing slopes (Pavlicek et al., 2003). Among 2,500 species recorded in "EC" I, several mainly eukaryotic model species (fungi, plants, and animals) have been studied in detail (Nevo, 1995; Derzhavets et al., 1996; Derzhavets et al., 1997; Nevo, 1997; Lamb et al., 1998; Grishkan et al., 2000; Kalendar et al., 2000; Korol et al., 2000; Michalak et al., 2001; Saleem et al., 2001; Dvornyk et al., 2002; Finkel et al., 2002; Nevo et al., 2005; Rashkovetsky et al., 2006).

The *Bacillus simplex* Model from "Evolution Canyon"

We have recently established a bacterial model population system of *Bacillus simplex* strains from "EC". In this section we describe the potential of this population to study adaptation and speciation in natural habitats, and we will relate this to modern thoughts in taxonomy.

Identification of the phylogenetic structure and its correlation to the habitat type. According to current bacterial systematics (Rosselló-Mora and

Amann, 2001; Stackebrandt et al., 2002), all members of the metapopulation of *B. simplex* (approx. 950 strains) belong to a single species (Heyrman et al., 2005; Sikorski and Nevo, 2005). However, the metapopulation shows a well developed structure of adaptation to the two spatially very close but microclimatically strongly contrasting slopes of "EC" (Nevo, 2001) (Fig. 1), which consists of several ecologically strictly separated groups or lineages (Sikorski and Nevo, 2005). This suggests that the evolution of the described *B. simplex* metapopulation lineages here follows a mode of evolution as described by the Stable Ecotype Model, according to which each lineage (ecotype) represents a biologically meaningful bacterial species. The metapopulation consists of two genomic lineages (GL): GL1 and GL2 (Fig. 2a). GL1 is primarily African-like, containing two "African" lineages A and C, and one "European" lineage B. GL2 is primarily European-like, consisting of a dominant "European" lineage E and a small "African" lineage D. The phylogenies deduced from the nucleotide sequences of parts of three genes (*gapA*, 654 bp; *pgk*, 426 bp; *uvrA*, 1,135 bp) of representative strains from each RAPD cluster and from both canyons (N = 21 to 27) clearly corroborate the RAPD clustering at the A–B–C versus D–E split and with very good congruence at the levels of the individual lineages A, B, C, D, and E. Lineage B shows an apparently ecologically driven nested substructure (Fig. 2b). An "African" lineage Ba is nested within "European" B, with strains from all six "African" stations (stations 1–3 of both "EC"s). This pattern suggests an evolutionary budding of Ba strains from B-like ancestors. A second nested pattern, Bae within Ba, is currently only suggested by RAPD patterns (Fig. 2a) and awaits further sequence-based confirmation.

The adaptation is driven by a strong slope-specific selective regime significantly overriding migration (between canyons and slopes) and stochasticity and resulted in the formation of several slope-specific phylogenetic evolutionary lineages, i.e., ecotypes (Sikorski and Nevo, 2005) (Fig. 2a, b). The well developed correlation between genetic clusters and the type of habitat makes this population highly suitable for studying the evolution of phenotypes that might have driven the evolutionary separation of the lineages under respective ecological pressures. (Sikorski and Nevo, 2005).

Quantitative expression differences of phenotypes among the observed putative ecotypes. As discussed above, it is not sufficient to identify the correlation between genetic groups and types of habitats – the correlations must be supported by ecologically relevant phenotypes. Up to now, we analyzed three phenotypes with different levels of potential references to the environmental stresses exerted by the heterogeneity of the slopes of “Evolution Canyon”. It is essential to include as many strains as possible in phenotypic studies, therefore, a subset of 131 strains was chosen in order to represent the evolutionary genomic lineages (ecotypes) introduced above. Due to the large diversity also within the evolutionary lineages, single strains might not necessarily reflect the characteristics of the lineage to which it belongs. The main abiotic factors in “EC” are solar radiation, drought, and heat (Nevo, 2001; Pavlicek et al., 2003). Two studied phenotypes refer directly to the above abiotic stresses. We studied the survival of bacterial cells after UV-C radiation (adaptation to solar stress) (Sikorski and Nevo, 2005). Also, we measured the ability of cells to replicate at high stressful temperature (adaptation to thermal

stress) (Sikorski and Nevo, 2006). The expression levels of both phenotypes could have evolved as a direct response to the different levels of solar radiation and temperature on the AS and ES of "Evolution Canyon". Thus, we expected strains from the AS to be more tolerant to UV exposure, and also to grow better at extreme high temperatures. A third phenotype is mutation rate. Although the genetic modifiers for different levels of mutation rates are only selected indirectly, it can be supposed that the genomic mutation rate is adjusted to a level that best promotes adaptation since mutations are the ultimate source of the genetic variation required for adaptation (Ishii et al., 1989; Sniegowski et al., 2000). The AS and ES expose different levels of environmental stress, which could indirectly cause different levels of mutation rates. One could hypothesize a higher mutation rate on the AS since this slope is generally regarded to be the more stressful slope (Nevo, 2001). We therefore determined the spontaneous mutation rate in fluctuation tests by using resistance to the antibiotic rifampicin as a phenotypic marker (Sikorski and Nevo, 2005). Below, we summarize the results on these phenotypes (Sikorski and Nevo, 2005; Sikorski and Nevo, 2006).

Mutation rate. We observe significant differences in the mutation rate between the evolutionary lineages (Fig. 2). In genomic lineage GL1, "African" lineage A shows a high mutation rate, whereas the "African" lineage C is fairly low. "European" lineage B and its derivatives express an intermediate level. In GL2, the mutation rate of the "European" lineage E is significantly higher than that of "African" lineage D. However, as a total, "African" strains do not differ from "European" strains in their mutation rate. Thus, we do see significant

differences between lineages, but we do not observe a direct correlation of the level of mutation rate with either slope type (Fig. 2) (Sikorski and Nevo, 2005).

UV-C survival. For the phenotype of UV-C survival, most differences among lineages within GL 1 are nonsignificant. Interestingly, "European" lineage E shows significantly higher UV survival than "African" lineage D. As a total, "African" strains do not differ from "European" strains (Fig. 2) (Sikorski and Nevo, 2005). The phenotype of UV-C survival is genetically most probably based on DNA-repair, such as excision repair of nucleotide (pyrimidine) dimers. However, DNA damages may also result from other environmental stresses (Friedberg et al., 1995). Thus, differences in the UV-C survival patterns among evolutionary lineages could be driven by a variety of environmental factors; but we do not have evidence to assume that the higher solar radiation on the AS is a major contributing factor (Fig. 2).

We could not identify a direct correlation of phenotypic expression levels of either mutation rate or UV-survival with either slope type. This, however, does not indicate that there are no environmental stresses affecting these traits. Soil is an extremely complex habitat and may contain a huge number of different ecological niches. Apparently, the ecological forces that drive the observed patterns of these phenotypes may not be directly related to the major abiotic forces of solar radiation, drought, and temperature at "Evolution Canyon", and might therefore have not been identified yet. However, we observed significant differences in both phenotypes among "African" and "European" lineages in the shape of their distribution curves (Sikorski and Nevo, 2005). This indirectly suggests that the UV-C survival phenotype is more affected on the AS, thereby

reflecting the possible influence of stronger solar radiation, whereas the mutation rate phenotype is more affected on the ES, probably reflecting stresses by the higher soil internal heterogeneity on the ES (Sikorski and Nevo, 2005). A similar phenomenon – the different ecological stresses exerted by the AS or ES affect different biological aspects of wild barley, *Hordeum spontaneum*, at “Evolution Canyon” – has recently been observed (Nevo et al., 2005).

Thermal stress. In contrast to the phenotypes discussed above, the phenotype of growth capacity at a high stressful temperature seems to be a direct response to the hotter temperatures on the AS (Fig. 2) (Sikorski and Nevo, 2006). The "African" GL1 lineages A, Ba, and C grow significantly better than the "European" lineage Bae, but not more than the "European" lineage B. Also, the GL2 "African" lineage D grows significantly better than its "European" sister lineage E. Thus, all individual "African" lineages grow (in most comparisons significantly) better than all individual "European" lineages. Taken as a whole, "African" strains grow significantly better than "European" strains (Fig. 2) (Sikorski and Nevo, 2006). This indicates a strong adaptive pattern of the bacteria to the environmental heat stress. Further analysis also indicates that the adaptive process to the hotter AS is still (Sikorski and Nevo, 2006). Moreover, we have evidence suggesting that temperature stress at “Evolution Canyon” is restricted to the AS, whereas ES strains are under no temperature stress at all.

Summarizing the adaptive evolution of *B. simplex* at "EC". Segregation of the *B. simplex* population in "African" and "European" lineages is strongly driven

by heat stress. In parallel, also other phenotypes, such as DNA repair (as estimated by UV-survival) and mutation rate, diverge across lineages. The factors that affect the two latter traits are yet unknown. The parallel existence of individual "African" and individual "European" lineages is most probably due to the enormous microheterogeneity within the slope soils itself, which allows for different ecological niches within the same slope type. These initial studies show the suitability of the *B. simplex* metapopulation at "Evolution Canyon" to study bacterial evolution in natural habitats. But moreover, it is an exemplary model system for tracing bacterial evolution and discussing traditional and modern concepts of species and speciation.

***B. simplex* and modern taxonomy**

The demarcations of current bacterial systematics (Rosselló-Mora and Amann, 2001; Stackebrandt et al., 2002) include all introduced *B. simplex* evolutionary lineages into a single species (Sikorski and Nevo, 2005). However, according to the metapopulation lineage concept of the Modern Synthesis, the evolutionary lineages should each be recognized as individual species because they show contingent species properties, which are generally accepted by many systematists outside of microbiology (Bock, 2004; de Queiroz, 2005a): they are ecologically distinct groups that are genetically cohesive and separately evolving. In the Stable Ecotype Model, each ecotype undergoes a series of periodic selection events during its long history of divergence from other such ecotypes (Gevers et al., 2005). Our data suggest that "African" lineages are currently undergoing periodic selection towards higher heat resistance, and

more bouts of heat adaptation are highly probable to come (Sikorski and Nevo, 2006). The open environmental system of "Evolution Canyon", particularly the "African" slope with its high heat, functions thereby as a motive force for further periodic selection and provides a good prognosis for future coexistence of "African" and "European" lineages of *B. simplex* by continuously driving their divergence.

Following the Modern Evolutionary Synthesis (Simpson, 1961; Mayr, 1969, 1982), the species concept is an integral part of evolutionary theory (Bock, 2004; de Queiroz, 2005a), rather than of systematics. However, identification of the species taxon based on criteria derived from a theory-based evolutionary species concept (e.g., as introduced by Frederick M. Cohan in this special issue) has been rejected due to pragmatic reasons although a more natural species concept would generally be welcome (Coenye et al., 2005). Our work on *Bacillus simplex* from "Evolution Canyon" exemplifies that current bacterial systematics is rather obstructive in understanding prokaryotic biology and evolution. We therefore argue for the development of a theory-based bacterial systematics, including deriving ways of how this could be made pragmatic and applicable.

Conclusion

The introduction of modern and theory-based frameworks of population dynamics in bacterial systematics, ecology, and evolution is just nascent but long overdue. Development, description, and elaboration of theory-based models of how bacteria might evolve is strongly initiated and facilitated through

thorough analysis of populations from nature (Gevers et al., 2005). Current approaches of mathematical/computational studies or of Experimental Evolution do not yet incorporate ideas from these theory-based frameworks, and it is not known if they will be able to do so. Therefore, further exploration and understanding of bacterial evolution, which must be embedded in modern thoughts of evolution and taxonomy, is indispensably linked to the study of natural populations.

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Figures

Fig. 1: The “Evolution Canyon” Model. (A) Schematic diagram, (B) cross section view of “Evolution Canyon” I, Lower Nahal Oren, Mount Carmel, (C) air view of “Evolution Canyon” I. Note the plant formation on opposite slopes. The green, lush, “European”, temperate, cool-mesic ES sharply contrasts with the open park forest of warm-xeric, tropical, “African-Asian”, savanna on the AS. In each “Evolution Canyon” seven sampling stations are designated: three on the AS (1-3), one at the valley bottom (4), and three on the ES (5-7).

Fig. 2: Evolutionary lineages in the metapopulation of *B. simplex* and their phenotypic characteristics of mutation rate, UV-C survival and high-heat stress resistance. Fig. 2a and 2b are adopted from Sikorski and Nevo (Sikorski and Nevo, 2005).

(a) Neighbor-joining tree (Jukes–Cantor distances) from RAPD data of approximately 950 isolates. Thin horizontal lines to the right of the tree indicate origin from the “African” slope (AS, light grey), valley bottom (VB, black), and “European” slope (ES, dark grey). Thick vertical lineages denote the strain grouping into evolutionary lineages A to E. (b) A phylogenetic tree of the evolutionary lineages depicting the consensus of neighbor-joining, minimum evolution, maximum parsimony, and maximum likelihood analyses of three different genes sequences from up to 27 representative strains (Sikorski and Nevo, 2005). GL1 and GL2 denote genomic lineages 1 and 2, respectively.

c) Box-Whisker-Plots of three phenotypes of the indicated evolutionary lineages and the total of "African" and "European" strains (numbers of strains are indicated in brackets). The open circle and filled square indicates mean and median values, respectively. The box shows the standard deviation, whereas the "error bars" depict the maximum and minimum values. Thin dashed vertical lines indicate the upper and lower quartil values. Light grey boxes denote "African" AS strains and dark grey boxes denote "European" ES strains. The growth values (growth at high heat stress) represent the integral of the growth curve between 60 and 540 minutes in liquid rich medium (Sikorski and Nevo, 2006). It turned out that the shape of growth curves at 43.25°C is so diverse that the growth response can not be quantified by the usually applied measure of generation time (Sikorski and Nevo, 2006). Growth at 30°C yields with all strains a growth value of aprox. 12-13, which reflects a generation time of approx. 35-40 minutes and an entry into the stationary phase after approx. 5 hours of culture duration (Sikorski and Nevo, 2006).

d) Table of statistical significant differences between lineages. Prior to the tests, we verified the assumptions (normal distribution and variance homogeneity) for applications. Since GL1 contains several evolutionary lineages, we applied methods for multiple comparisons of the mean values. * Kruskal-Wallis H-Test, ** one-way ANOVA F-Test, *** post-hoc Tukey-Kramer, **** post-hoc Bonferroni, *****Mann-Whitney U, n.s = non significant.

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Figure 1:

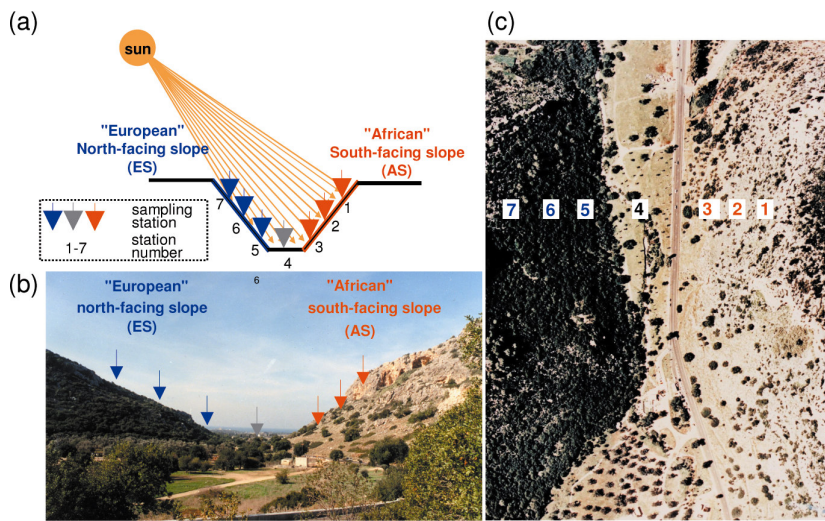


Figure 2:

