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REVIEW ARTICLE

Selection of very small differences in bacterial evolution

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Summary As the Science of Biology is constantly undergoing change due to new discoveries and advanced techniques it is essential that a systematic study of the environmental causes of natural selection on microorganisms be conducted. Very small phenotypic differences among individuals within bacterial populations arise as a result of spontaneous genetic variation, but the evolutionary importance of these small changes is frequently considered to be non-significant. Recent in vitro experiments indicate that efficient selection of these very small differences may take place in environmental compartments where a particular intensity of the selective agent is exerted. Model studies based on competition between bacterial populations only differing in one or two amino acid changes of a detoxifying antibiotic enzyme (e. g. β-lactamase) have shown that at a narrow range of antibiotic concentrations the variant population is strongly selected over the original type, despite the extremely low phenotypic differences in antibiotic susceptibility. These selective concentrations are expected to occur in precise environmental compartments (selective compartments). Due to the high frequency of structured habitats in natural environments, the intensity of selective agents is commonly exerted along certain gradients. Each one of the points forming these gradients (or intersection among gradients) may have a particular selective ability for a specific genetic variant. Considering the environment as a composition of an extremely high number of specific selective compartments may help to understand the existence of high levels of genetic variability in natural bacterial populations. This may be one of the clues towards the unraveling of bacterial evolution.

Key words Experimental evolution · Bacterial selection · Antibiotic resistance Antibiotic concentration · Bacterial environment

Bacterial evolution depends on the generation of bacterial genetic diversity and on the efficiency of the selective processes in fixing a substantial number of bacterial variants. It is generally conceived that diversity-making genetic systems offer a huge quantity of variation, but that selective environments exert a sweeping effect reducing genetic diversity. This review looks at the importance of environmental selective heterogeneity for the maintenance of bacterial genetic variation. A significant part of genetic variation leads to very small changes in the phenotype. It is essential to consider whether these small differences are important in the selective (and consequently in the evolutive) process. If they corroborate that selection occurs, then the consideration of the environment as a variation repressor may be challenged, or on the contrary, the environment as facilitator of variation can be reconsidered.

Very Small Differences Selection

Very Small Differences Selection (VSDS) requires, obviously, the existence of very small differences between individuals (in respect to a potential trait) and a "weak" selective process. For a selective pressure to be considered as such, it should not be able to induce selection among individuals with large differences between them. To a certain extent, VSDS is able to shape the evolution of a complex biological system when a fine-grained variation between individuals exists. In *Darwin's Dangerous Idea* [11], Daniel C. Dennet commented on a significant discussion between philosopher of biology Kim Sterelny and geneticist Richard Dawkins, the celebrated author of *The Blind Watchmaker* one of the main rhetorical points (speaking about a protostick insect) was as follows: "Come on, are you really trying to tell me that 5% like a stick really matters compared to 4%?" Clearly there is a commonsense tells us to be sceptical about taking into account very small differences. Indeed, the main conclusion of the VSDS hypothesis is that "weak" selective processes are indeed "strong" selective processes when acting on VSDS. Weak selection may in fact efficiently change gene frequencies. Several surveys on enzyme polymorphisms in microbial species reveal that many loci have a multiplicity of alleles. The selection that causes a multiple allele polymorphism is probably of the VSDS type. The microbial world is a particular area of nature where VSDS may be particularly important. In many instances, large population sizes are required for the optimal effect of weak selection

pressures, so that it may prevail in the face of genetic drift. The VSDS process is reviewed here under the hypothesis that the environment is largely composed by quantitative gradients composed of fine-grained points with specific selective effects on particular microbial variants (forming a field of fine-grained variation).

Genetic variation and the environmental variation

The existence of high levels of genetic variability in natural populations remains one of the central facts confronting evolutionary biologists. In particular, microbiologists are faced with daily evidence of the huge adaptive and evolutionary possibilities of bacteria, based on the combination of immense population numbers and overwhelming genomic diversity. The understanding of the mechanisms which account for such variability and evolution is the main goal of evolutionary biology. Interestingly, many experimental studies on bacterial evolution have recently been carried out to address general issues in evolutionary biology.

For bacteria and for any other living organism, variability is a pre-requisite for selection and evolution; but selection tends to reduce the diversity of variants. For instance, periodic selections sweep out any variations that might be present in the original population. This phenomenon has been described as a competing relationship between selection and entropy increase. The maintenance of biological evolution is assured as variation is constantly recovered by organism-related diversity-making processes such as mutation and acquisition of foreign DNA, and also by the exposure to alternative environments (migration).

The main concept reconsidered in this review is that maintenance of biological diversity in organisms, including very small differences among individuals, depends on the variety of selective pressures in the environment. If the variation of the organism (the potentially selectable traits) is confronted with a correspondingly high diversity in selective forces, the final biological variability is expected to be maintained. Surprisingly, as Robert N. Brandon argued, the concept of environment has been largely ignored in the theory of natural selection [8, 9]. The origin of the diversity of environmental selective forces has been less explored than the origin of the cellular mechanisms leading to the biological diversity. As Daniel E. Dykhuizen recently stated, a systematic study of the environmental causes of natural selection is now required [12]. The concept of environmental selective compartments, reviewed in this paper, may contribute to fulfilling this objective.

The environment as composed by potential selective compartments

The diversity of environmental pressures appears to be related to habitat compartimentalization. It is expected that compartimentalized environments can lead to spatial genetic polymorphism. But what is meant by a compartment? The answer is relatively clear in complex heterogeneous environments, where a compartment means a qualitatively different sub-environment in which particular selective forces are exerted. Could we also conceive of compartments in qualitatively homogeneous environments? This point is essential for the purpose of this discussion. In this type of simple environment, the circumstance that may be able to create local differences is the formation of concentration gradients. Gradients are particularly evident in physically structured habitats, producing new spatial heterogeneities. In a continuous gradient of a given component, its concentration at both extremes of the gradient may be sufficiently different to view these extremes as separate compartments, where different selective forces are exerted. Imagine salt, light or temperature gradients, selecting halophylic, photophylic or thermophylic microbial variants only at high concentrations. But, for instance, in the last case, intermediate temperatures will select mesophilic and lower temperatures cryophilic variants, indicating the possible presence of three selective compartments. But the number of selective compartments is probably higher; one can readily imagine the selection of a given microbial variant with an optimum growth rate in the range 40° to 42°C, but not under or over this range. Thus, within a gradient, a selective compartment can be defined as the “concentration” (or range of close concentrations) able to select a particular geneticvariant [3, 4].

The selective compartments along a natural gradient can change both in space and in time; in this way they can alter the potential selectivity for the different genetic polymorphisms present in natural biological populations. For instance, a given concentration of an environmental agent may have different selective effects on an organism depending upon the time that of exposure. After a given period of time a new set of selective compartments may arise. Obviously, gradients submitted to frequent fluctuations may dissipate the selective power of each compartment, as the process of selection is dependent on a critical period of time, which is variable for each pair of selective agent and selectable population. Further generation of selective compartments can occur as a result of interference between gradients, which results in compartments whose selective activity depends on the combined action of more than a single agent [3].

On the other hand, as the population structure is modified by the selective process, the gradient itself may be modified as well.

Eventually, such modification may "enlarge" the existing selective compartments, leading to an "in-chain reaction" phenomenon. In other cases, new selective compartments could be created, for instance, by the excretion of allelopathic substances to kill competitors. This strategy of enlargement or production of selective compartments is certainly one of the characteristics defining the evolutionary possibilities of living beings. The complementary response is to increase genetic variability by promoting frequency-dependent selection to obtain better-fitted organisms, able to compete (to be selected) in pre-existing unexploited compartments or in newly created ones. To a certain extent, both strategies may be linked. It is possible that during the growth in a given compartment the organisms may create secondary selective compartments, which enrich subpopulations with an increased mutation rate. That could be the case for the excretion of signalling molecules at high bacterial densities which regulate the expression of genes involved in stationary phase. Some of these genes are involved in population shifts favoring aged subpopulations and in altering the number of mutational events.

In this way, the number of selective compartments along a given gradient depends upon the diversity of selectable variants. The response to the question "how different must a variant be from the original organism in order to be selected?" is indeed the same as that to "how different should the concentration between two points in a gradient be in order to constitute different selective compartments?" As has been mentioned, physically structured habitats may maintain gradients more efficiently, and, consequently, they constitute selective compartments. This is probably the reason for the acceleration in evolutionary diversification that is observed in structured environments. A final corollary of these views is the following: the selectability of a given variant can only be ascertained if it is tested in the corresponding selective compartment. It is possible, of course, that two different variants may find apparently equally suitable alternative solutions to a given environment. To ascertain that the solutions really are "equally suitable", competition experiments between both variants at precisely defined compartments are advisable, as they may eventually lead to the further distinction of sub-enviroments.

Selection of antibiotic resistant variants in antibiotic selective compartments

The bacterial development of antibiotic resistance is one of the best documented examples we have of contemporary biological evolution. The role of antibiotics as agents involved in selection and evolution of antibiotic resistance is beyond any doubt. Antibiotics used in chemotherapy create, in the human body, a high diversity of concentration gradients. These gradients are due to pharmacokinetic factors, such as the different diffusion rates into various tissues, metabolization, local inactivation, or variation in the elimination rate from different body compartments. The direct effect of microbes of the normal or pathogenic flora, particularly (but not exclusively) if they possess antibiotic-inactivating enzymes, also contributes to the gradient formation. In general, after antibiotic administration, high antibiotic concentrations will be confined to small and ephemeral selective compartments, and low concentrations are expected to be distributed during a longer time in large selective compartments. Therefore, most bacterial populations in the human microflora probably face a wide range of antibiotic concentrations after each administration of the drug. Because the spontaneous genetic variability of microbial populations also provides a wide range of potentially-selectable variant subpopulations, it is appropriate to determine the selective compartments where a particular antibiotic concentration is able to select one or other of these particular subpopulations.

Any antibiotic concentration can potentially select a resistant variant if it is able to inhibit growth of the susceptible population but not that of the variant harboring the resistance mechanism. In other words, a selective antibiotic concentration is that which exceeds the minimal concentration able to slow the growth rate or eventually to kill of the more susceptible population, but not that of the variant (less susceptible) population, even if it is very close. At antibiotic concentrations able to completely suppress the growth of both susceptible and variant populations, selection of the variant is not expected to occur. The same applies when the antibiotic concentration is below the local MICs of both populations. Therefore, the selection of a particular variant may happen *only* in a narrow range of drug concentrations. For instance, in some systems where the "susceptible" cells outnumbered the variant "low-level resistant" population, the selective antibiotic drug could select these bacterial variants only at a low concentrations [2–4].

Selection of variant β-lactamases

In vitro experimental systems are able to confirm this concept. For such a purpose, we studied the antibiotic-mediated selection of bacteria harboring wild and variant β-lactamases. β-Lactamases are enzymes produced by a variety of pathogenic microorganisms, able to inactivate (detoxify) β-lactam antibiotics, such as penicillins. For instance, ampicillin is inactivated by the β-lactamase TEM-1, and the producing organisms are resistant to this drug. To counteract this resistance, new β-lactam antibiotics "resistant" to β-lactamase TEM-1 were developed by the pharmaceutical industry, as the third generation cephalosporins (cefotaxime is an example),

that suppress the growth of bacteria carrying TEM-1. In a relatively short period of time, bacteria have counteracted this challenge by the production of variant TEM-1 β-lactamases, now able to hydrolyze the recently introduced cephalosporins, in a typical example of a relationship between enzyme activity and bacterial fitness. To our surprise, the study of the protein sequence of variant TEM enzymes found in microorganisms involved in infections refractory to cefotaxime therapy frequently showed that *several* amino acids were changed with respect to the original TEM-1 sequence. This suggested that a cryptic evolution may have occurred in hospitals, following a subtle selection of single mutants which gave rise to populations where a second mutation appeared. These were in turn selected, and the repetition of the process produced many of the more efficient antibiotic-inactivating enzymes. The same type of process has been described for other genes, such as *bgl*. Why were we, medical microbiologists, unable to detect the first variant enzymes? The reason was that these variants had only a minimal increase in the ability to inactivate the new antibiotics. For instance, in conventional susceptibility testing essays, bacteria harboring the original TEM-1 enzyme were inhibited by 0.03 μg/ml of cefotaxime; those with a "first" TEM-12 variant, resulting from the single substitution of arginine for serine at the position 164, were inhibited by only 0.08 μg/ml of this drug. This minimal increase was considered meaningless in terms of therapy or evolution of resistance, since the antibiotic concentrations in the patient were expected to be sufficient to suppress the variant. In reality, the organism harboring the TEM-12 variant was indeed selected. Shortly after, the acquisition of a new mutation, now replacing glutamic acid for lysine at the position 240, produced the more efficient enzyme TEM-10 which significantly increased the ability of the host bacteria to survive in relatively high cefotaxime concentrations. Thus, it created clinical problems for the therapy of human infections.

How could the "first variant" TEM-12 harboring population be selected? According to the hypothesis discussed here, that occurs within a particular selective compartment. In this case, selective compartments, responsible for this type of limited selection, could be considered as the virtual space or niche in which a precise concentration of antibiotic provides a punctuated selection of a particular resistant bacterial variant. The antibiotic concentration exerting such an effect has been designated as the "selective antibiotic concentration" [4]. The selective compartments can be reproduced using in vitro models. Using directed mutagenesis technology, we prepared a collection of isogenic *Escherichia coli* strains harboring TEM-1 (wild enzyme), TEM-12 (first variant, with one single mutation) and TEM-10 (two mutations) [6]. Double and triple mixtures of these strains were prepared, at the respective 100:10:1 proportions, and then challenged during 4 hours by different cefotaxime concentrations, corresponding to different environmental compartments within a gradient. The antibiotic was enzymatically degraded, and the surviving cells transferred to antibiotic-free medium, and the proportion of cells containing the wild and variant enzymes were analyzed in these cultures after overnight growth. In several independent experiments, the same result was obtained: TEM-12 was selected over TEM-1 *only* at a narrow range of antibiotic concentrations (0.008 to 0.06 μg/ml), which correspond to the TEM-12 selective compartments under the experimental conditions. In triple mixtures, the selective compartments for the TEM-10 variant were identified at higher antibiotic concentrations. Essentially the same type of result was obtained in another model, using this time mixtures of *Streptococcus pneumoniae* populations showing different levels of susceptibility to several β-lactam antibiotics [18].

In short, these experiments served to illustrate the basic concept that a particular variant is selected in a particular selective compartment within an environmental (antibiotic) gradient. An additional complexity of the system results from the interference between gradients, creating new bidimensional frames that generate new selective compartments. In the case of antibiotics, that may occur during combined or fluctuating antibiotic therapy schedules. We have shown [7] that some supposedly "neutral" mutations in TEM-1 β-lactamase (such as the replacement of alanine for threonine in position 237) have probably been selected in this type of compartment, as a result of the optimization of the enzyme to deal with combined antibiotic challenges (ceftazidime + cefotaxime). This type of result was predicted by earlier studies on the evolution of selective neutrality. In conclusion, the significant diversity in β-lactamases may express the diversity of selective antibiotic compartments that occur during therapy. New compartments were produced from the introduction of new antibiotics or therapeutic schedules that resulted in the diversification of TEM β-lactamases; this is certainly an elegant testimony to the flexibility of biological systems and the power of natural selection.

Environmental selective landscapes

The classic study of Albert F. Bennett and Richard E. Lenski on experimental evolution of bacterial populations challenged by different temperatures can be reinterpreted as a similar example of the selective importance of environmental compartments. In this study, replicate bacterial populations were propagated for 2,000 generations at constant temperatures of 32, 37 and 42°C, and then the increase in fitness at different temperatures was tested [15]. Three types of resulting "thermal specialists" were selected, with improvement relative to the common ancestor precisely at temperatures that closely corresponded to the constant temperatures at which they were propagated, showing little or no improvement only a few degrees away. These temperatures can be well considered as selective compartments within a thermal gradient. Similar to the case of combined antibiotic selection commented at the end of the former paragraph, a fluctuating challenge with different temperatures (32 and 42°C) produced a different bacterial variant that was a "jack of all temperatures but master of none" [16]. It can be considered that this variant was selected in a new compartment defined by this particular combination of environmental features.

The correspondence between selective compartments and selectable variants could be a way of describing the selective

landscape of a given environment. The idea of measuring the environment using organisms as measuring instruments ("phytometry", when plants were used) was advocated a long time ago (1924) by Clemens and Goldsmith [10] and more recently by Antonovics et al. [1]. The last authors and Robert N. Brandon discussed the concept of "selective environments", expressing environmental differences across heterogeneous "regions" [9]. Selection occurs when differential adaptedness to a common selective environment leads to differential reproductive success. According to Kauffman's version of Wright's classic concept, the distribution of fitness values over the space of genotypes constitutes a fitness landscape [14]. What we emphasize here is the close correspondence that should exist between organismal fitness landscapes and environmental selective landscapes. Fitness (or adaptive) landscapes representations are excellent ways to represent the possibility of selection of a particular variant under particular selective conditions. Eventually, you can get an isolated peak of selection when you are experimentally scanning a wide range of selective concentration. This may occur for a variant with a very small difference in relation with the surrounding genotypes; if this occurs, you have found the needle in the haystack, despite the "common sense" prediction that such difference will be "practically" invisible to natural selection. An ideal scanner of selective conditions (for instance, different antibiotic concentrations) may indeed resemble a tuning device which selects a certain radio frequency emission. Under or over such a frequency (the antibiotic selective concentration), the emission (the particular variant) is lost (selection does not take place). The saddle between the concentrations inhibiting the susceptible and resistant populations is like the frequency signal recognized by the selective concentration.

Note that the compartment hypothesis here discussed was only applied to the understanding of the selection of variants, but may eventually contact with the broader essentialist (aristotelian) method of explaining variability, that assumes that there is some finitely stable conditions which all and only the members of a group (for instance a species) satisfy. These conditions may reflect a unique relationship of a genome and a particular environment. There is a possibility to conceive an enviromental equivalent ("envirome") of a particular genome. In this sense, the "envirome" can be defined as the assembly of environmental features which have contributed to the establishment—the selection—of a unique genome [5].

Evolutionary importance of Very Small differences

In organisms with very large effective population numbers, it seems highly probable that the normal pattern for natural selection would be to act on small fitness differences. According to conventional wisdom, in very modest margins differences in actual survival and reproductive success may be attributed only to chance. Nevertheless, one of the key developments in the process of modeling biological systems is the so-called individualbased (or individual-oriented) population models. These are based on the stochastic birthdeath process which, as it was said by Haefner [13], ultimately is based on random walks or Markov chains using probability theory developed in the XIX century. Interestingly, the basic concept is that individuals within a given population influence the evolution of such a population despite apparently minor contributions to the community. Therefore, small variations among individuals can have dramatic effects on the ultimate state of the population. Such a conclusion is certainly supported by the consideration that selective compartments in environmental gradients are eventually able to differentiate among variants with tiny fitness differences. Among scientists not directly involved in evolutionary biology (and in particular among microbiologists), there is a common intuitive opinion that any efficient selective process requires "significant variations" both in the phenotype and in the selective environment. For instance, a widespread assumption is that "if the phenotypic effect of a mutation is low, its contribution to the selective advantage should be similarly low". The evolutionary significance of some recently isolated β-lactamase mutants was not clear to the authors, "since we do not know how small an effect constitutes a selective advantage". Indeed, this question can only be answered in relation with the identification of the corresponding selective compartment. In this case, it is quite possible that the selective importance of this obscure mutation could be only ascertained at a given β-lactam concentration (but not *over* or *below* this concentration).

Similarly, "minor" environmental variations are also frequently qualified as irrelevant. For instance, small quantities of antibiotics released in the soil by producing organisms, or present in foodstuffs as a result of antibiotic-supplemented feed used to enhance animal growth, have been considered as unsufficient to produce any significant biological effect. Some results discussed in this review suggest that very small antibiotic concentrations may produce selective compartments where low-level resistant bacterial variant populations could be amplified, perhaps feeding the evolutionary process leading to clinical resistance. Many other secondary metabolites only reach low concentrations in the environment, but they may also be extremely relevant in selection of particular populations.

Some biotechnological applications may result from the consideration of the selective effect of small concentrations of biologically active substances. Many useful substances of this type may have been overlooked in pharmaceutical screening programs due to the absence of sensitive detection tests. Now we can easily imagine a detection system based on the selection of low-level responder organisms by small quantities of active substances acting on a adequately chosen mixture of responder and non-responder bacteria.

In general, the experimental selection of some unknown genetic variants in a given population may be extremely difficult without extensive testing along a wide range of concentrations of the selective agent. Experiments of selection carried out at a fixed concentration will enormously reduce the chance of detecting bacterial variants, and this may give a false image of genetic homogeneity for the studied population. In summary, the

evolutionary significance of a given mutant can be only evaluated in its selective environment. Also, as Richard Lenski pointed out [17], if subtle selection for some particular variants may occur only in very precise compartments, then this may explain how double mutants may reach high frequency without invoking the notion of "directed mutation".

Considering the extreme diversity of selective compartments and gradients in natural environments, may help explain the maintenance of a tremendous genetic variation. It is certain that the analysis and description of selective compartments in the real world, with its "myriad complexities", will be an extremely difficult task. In any case, the improvement of our methods and strategies is essential to approach such an objective, as there is a growing need to predict the results derived from the full range (from subtle to catastrophic) of mankind's impacts on the environment.

Gottfried Wilhelm Leibniz, the person who most contributed both in philosophy and mathematics to the understanding of a continuum as composed of a multiplicity of qualitatively different units of activity, visited Antony van Leeuwenhoek at his home at Delft in 1676. The time has perhaps arrived for microbiologists to return this visit to Leibniz and to discuss with him how to apply the basic concepts which permitted the discovery of *Calculi Differentialis* to the elucidation of the clues of microbial evolution. The central idea is that subtle (infinitesimal?) environmental changes may have sufficient selective efficiency to provoke at least tiny bits of progress, although sometimes it is much more than a bit. Because of that, the exploration of the processes leading to the selection of (even) very small differences may be crucial for the understanding of microbial evolution.

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