

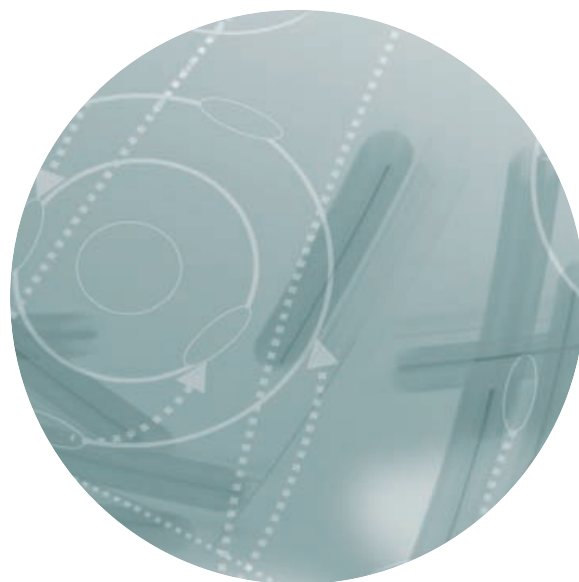
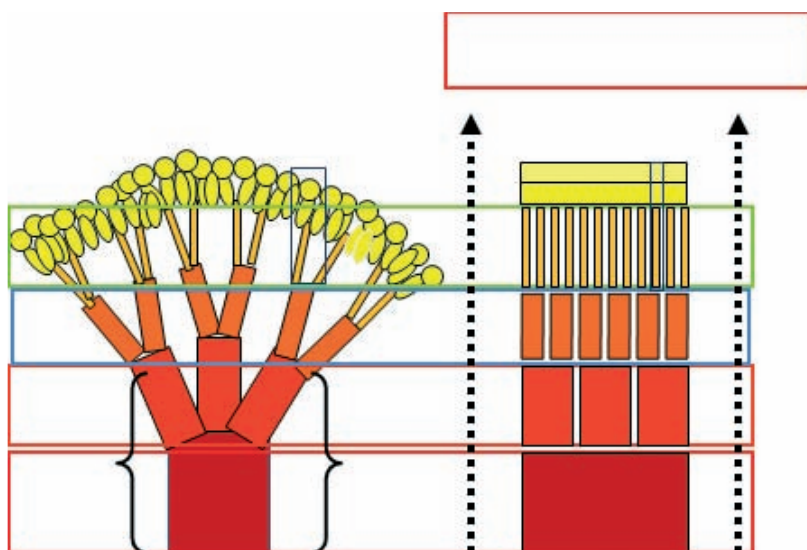
# CMI

# CLINICAL MICROBIOLOGY AND INFECTION

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**Environmental changes, microbial systems and infections**

Guest Editors: F. Baquero, R. Cantón and C. Nombela



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# CLINICAL MICROBIOLOGY AND INFECTION

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## Environmental changes, microbial systems and infections

### GUEST EDITORS

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# Everything depends on everything else

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## Abstract

In physics the concept of entanglement is well established and it has become increasingly apparent that all levels of biological organization (communities, organisms, cells, metabolism) consist of mosaics of interactive networks. There is a universe of bioactive microbial chemicals that have so far only been considered for their therapeutic applications; for example, the environmental roles of antibiotics have been little investigated. At sub-inhibitory concentrations, so-called antibiotics have been shown to modulate bacterial functions in subtle ways; they behave more like signals than toxins. It is proposed that networks of microbial cell signalling are primarily based on the interactions of low molecular weight compounds with macromolecular receptors; studies of the nature of these signals will reveal important information on the functions of microbial communities.

**Keywords:** Antibiotic resistance, antibiotics, cell–cell interactions, microbial communities, natural products, networks, quorum sensing, resistome, signalling, small molecules

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## Introduction

The title of this article is a translation of a credo of the Haida people, an important Northwest Indian band who live on the Queen Charlotte Islands off the coast of British Columbia. It refers to the interdependent and interactive relationship between people, animals, and their natural environment, a concept that governs their lifestyle. The Haida anticipated the theory of random networks in biology. A more global concept of interdependence, the Gaia principle, was later expounded by James Lovelock. I wish to discuss the nature of the interconnectivity between and within microbial cells, their hosts, and the environment, and the fundamental role that bioactive small molecules play in the kingdom of microbes.

## What are antibiotics?

Organic chemists have long been interested in natural products, and their investigations have contributed to many advances in chemistry, especially the total synthesis of complex molecules with many chiral centres. Natural products

include an amazingly wide range of aliphatic, aromatic and heterocyclic products of microbes and plants, all of which are, by definition, bioactive molecules (although the bioactivity remains undetermined in most cases); many of these compounds are vital in the treatment of human diseases to this day. The discovery by Alexander Fleming and Selman Waksman of bacterial and fungal small molecules with antimicrobial activity was the foundation of the modern pharmaceutical industry; the introduction of penicillin and streptomycin completely transformed medicine and ushered in the 'golden age' of antibiotics. For the first time in human history, infectious diseases could be cured. This is rightly considered to be one of the 'miracles' of the 20th century. Unfortunately, during this time, the study of therapeutic functions dominated research, and scant attention was paid to the natural roles of these bioactive small molecules.

In 1942, Waksman proposed the definition of an antibiotic: a microbial product that kills or inhibits the growth of other microbes. However, it is now clear that this definition applies to only one aspect of the biological activity of microbial small molecules. They act in a concentration-dependent manner to modulate a wide range of metabolic reactions in environmental, clinical or laboratory situations. Waksman revised his definition later, in part because of the realization that the bacterial world is not one enormous battlefield of competing forces. It is now known that the number of different types of organic compounds that make up the natural small molecule world is enormous, quite possibly ten times greater than the number of living species. They are part of a biospheric interactome that links all cells and their

components; small molecules add additional levels of complexity to systems biology.

In recent times, with increasing knowledge of the diversity and complexity of the microbial world, the possibility that small organic molecules may play significant roles in the maintenance of microbial communities of terrestrial and marine environments has become relevant. Experimental advances, especially in the identification and study of transcriptomes and proteomes, have revealed many unsuspected molecular interactions in cells; cellular metabolism is not a series of independent linear pathways but consists of a series of hierarchical, connected networks [1]. One feature of many of these interactions is the involvement of small molecule signals and effectors.

### Signalling and communities

In the bacterial kingdom, the concept of intercellular signalling for the maintenance of multicellular communities and regulation of their social behaviour is well known, as are the phenomena of quorum sensing and the different roles of diffusible molecules over concentration gradients [2]. The ubiquity and chemical diversity of low molecular weight compounds (<5000 Da) produced by most microbes is consistent with this model.

Individual strains of large bacterial families such as the *Actinomycetes* (the source of many antibiotics) have the genetic capacity to synthesize more than 20 structurally different compounds [3]. Most of these compounds have not been isolated or characterized; their existence is inferred from the presence of specific biosynthetic gene clusters in their host genomes. Those that have been isolated are usually identified functionally by their antibiotic activity in the laboratory, with no consideration of their natural roles at reduced concentrations. In fact, the evolution of natural small molecules may be primarily associated with the survival of communities rather than the survival of the fittest. Their activities as therapies for a variety of human and animal diseases are consequences of their use in an entirely different, artificial context.

Most bacterial communities are very complex and exist as consortia of large numbers of different genera and subfamilies. One gram of soil contains upwards of 1000 different bacterial strains; the human gastrointestinal tract (microbiome) is inhabited by some  $10^{13}$  microbes with 1000 or more phylotypes. The human oral cavity contains as many as 300 different types of bacteria [4,5]. Intercellular and intracellular signalling is critical to the functioning of such community structures during fluctuating environmental and

nutritional states. It is probable that a collection of chemically diverse small molecules control these communities and their interactions with their environments, such as epithelial and buccal cells; the compounds act in a concentration-dependent manner and rarely (if ever) attain severely static or cidal concentrations. It has been suggested that the interactions constitute a homeostatic process, but the nature and complexity of the communities are as yet too ill-defined to permit this conclusion.

### What are the receptors?

The notion of a world modulated by small molecules implies that there must exist a large number of specialized receptors responsible for many types of extant small molecule interactions. One can envisage at least three different classes of signalling processes (Fig. 1); there are probably more. The best studied are those involving cellular receptors linked to two-component regulatory systems, such as the well-studied quorum sensing autoinducers that are found in many microbial genera. Quorum sensing regulates a multitude of different behaviours, including metabolism, motility and swarming, virulence, luminescence, development, antibiotic production, and horizontal gene transfer. The chemistry of the autoinducers encompasses many diverse structural types, and over 100 different molecules have been identified. In addition, multiple autoinducers made by the same bacterium can participate in cross-activation with different receptors; small molecule *antagonists* of autoinducer activity have also been identified [2]. Among their many functions, some autoinducers have antimicrobial activity, e.g. the quinolones made by *Pseudomonads* [6].

The antibiotics, another large class of bacterial small molecules, have a similar wide range of activities but operate by different mechanisms. The natural products used as

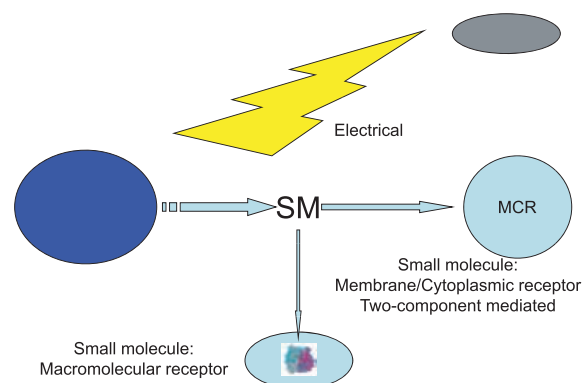


Fig. 1. There are (at least) three types of interbacterial signalling.

antibiotics act by binding to specific receptors that are embedded in the cell macromolecules involved in replication, transcription, translation or cell envelope formation. Each of these complex structures possesses many potential receptor sites for bioactive small molecules. Different regulons may be affected by different chemical classes. For example, the ribosome is a huge RNA–protein complex with numerous associated protein factors. Studies of small molecule binding to ribosomes (in the context of antibiotic mode of action studies) have revealed dozens of structurally specific receptors and ligand–receptor interactions, each of which can lead to modulation of distinct aspects of the function of the macromolecule in the cell [7]. It is likely that many more receptors exist within the ribosome structure; recent mutational analyses indicate a number of potential receptor sites on the large ribosomal subunit [8]. Evidence for discrete effects of these interactions comes from transcription studies with subinhibitory concentrations of different ligands, showing a range of responses due to binding at subinhibitory concentrations to different receptors within the structure [9]. Similarly, cell wall synthesis regulons are influenced by a number of small molecules [10]; for example, the regulation of enzyme activity in bacteria has long been identified as mediated by the specific binding of bioactive small molecules to proteins, DNA, or RNA (e.g. allosteric activators, repressors, and riboswitches) [11].

## Resistance/recalcitrance

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Internal cellular modulation (control) of the binding of low molecular weight ligands to macromolecular receptors can be brought about by enzymic modifications of the ligands, receptors, efflux, etc., which are common reactions in bacteria. These processes affecting ligand–receptor interactions define the resistome, as many of the chemical modifications have been identified as putative resistance mechanisms [12] and, when expressed independently in heterologous hosts, protect pathogenic bacteria against the activities of antibiotics.

Various modulation reactions may be converted into resistance mechanisms by horizontal gene transfer and overexpression in heterologous cytoplasm, e.g. the bacterial pathogens. Potential resistance mechanisms are widespread and have been revealed by gene knockout studies in environmental strains, etc. [13]. The situation is probably much more complex, as most single mutations in a gene result in disruptions of network activity. For example, mutations to antibiotic resistance in ribosomal protein genes are known to cause significant variations in cell metabolic phenotypes. Completely silent mutations may be rare. The soil and other

resistomes do not reflect the array of functions found in the clinic, although many are considered to have been recruited and disseminated by horizontal gene transfer.

## Plants—rhizosphere

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Microbes are not the only living organisms that make bioactive small molecules. The plant world is replete with such compounds, representing many structural types and biochemical activities [2,14]. As with the antibiotics, many are considered to be defence or repellence mechanisms. This may be only part of the story, as it has been shown that plant compounds can influence bacterial signalling and, conversely, bacterial compounds have significant transcription effects in plants. Work in my laboratory using bacterial reporter libraries has shown that a variety of plant-produced chemicals cause transcription modulation, confirming that environments like the rhizosphere are complex, interconnected, pro-eukaryote communities [15].

## Conclusions

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Small molecule signalling is an important field of study, and much work is needed to decipher the nature of the messages. It is likely that not all will be amenable to study in the laboratory, and in some cases it may be difficult to distinguish between specific signalling, broadcasting, and just plain noise.

A better understanding of the small molecule biology of different environments could be turned to advantage for the discovery of novel small molecule ligands, receptors and reactions useful in clinical (and industrial?) applications. For example, the identification and examination of foci of cell–cell signaling in soils may permit the direct identification of the signal origins from induced responses in reporter strains and provide microbial sources of novel classes of bioactive small molecules. Such approaches may eliminate much of the heuristic (hit-or-miss) aspect of natural product drug discovery. Small molecules are critical components of biological systems, and as has been proposed [16], it is time that they be included as part of the ‘central dogma’ along with DNA, RNA, and protein.

## Transparency Declaration

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# Environmental stress and evolvability in microbial systems

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## Abstract

The sustainability of life on the planet depends on the preservation of the existing microbial systems, which constitutes our major “biological atmosphere”. The detection of variations in microbial systems as a result of anthropogenic or natural changes is critical both to detect and assess risks and to programme specific interventions. Changes in microbial systems provokes stress, probably altering the local evolutionary time by changing evolvability (the possibilities of microbes to evolve). Methods should be refined to properly assess diversity in microbial systems. We propose that such diversity estimations should be done on a multi-hierarchical scale, encompassing not only organisms, but sub-cellular entities (e.g. chromosomal domains, plasmids, transposons, integrons, genes, gene modules) and supra-cellular organizations (e.g. clones, populations, communities, ecosystems), applying Hamiltonian criteria of inclusive fitness for the different ensembles. In any of these entities, we can generally identify, in a fractal manner, constant and variable parts. Variation in these entities and ensembles is probably both reduced and increased by environmental stress. Because of that, variation in microbial systems might serve as mirrors or symptoms of the health of the planet.

**Keywords:** Anthropogenic changes, environment, evolvability, stress, systems

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Who can explain why one species ranges widely and is very numerous, and why another allied species has a narrow range and is rare? Yet these relations are of the highest importance, for they determine the present welfare, and, as I believe, the future success and modification of every inhabitant of this world. (Charles Darwin, *On the Origin of Species*, 1859)

The question that we are examining in this short article is as follows: what are the reasons why environmental stresses, either natural or anthropogenic, might influence the evolvability of microbial entities? ‘Evolvability’ is the property of any biological unit to produce genetic variants of itself—either by mutation, internal recombination, or recombination with foreign DNA—so that one or more of these variants provides a direct or indirect fitness advantage over the ancestral biological unit. Evolvability is a selectable trait that is defined by rates of genetic change [1], and is therefore the precondition for genetic diversification and for biological diversity at large. Biological species result from a diversification process. The notion of species was critical in ascertaining the diversity of

biological beings. In 2007, we commemorated the 300th anniversary of the birth of Carolus Linneus, on 23 May 1707. The basic concept that he applied in establishing the notion of species (he used the term ‘varieties’) was hierarchical: species constitute fixed variants within something that remains constant, the genus. Indeed, the notion of species is a tool to denote *both* the units of classification and the units of generalization, i.e. to establish the fundamental groups of organisms according to which explanatory and predictive generalizations can be formulated [2]. This concept constituted the first tool for the systematic estimation of biological diversity.

The Linnean scheme is still used to classify all life forms, including bacterial organisms. But something seems to be wrong with the current concept of bacterial species—only about 5000 prokaryotic species have been described. Although The Global Biodiversity Assessment Program ‘suspects’ that there are more than a million species of prokaryotic organisms, the figure of 5000 is seemingly inconsistent with the one million *known* species of arthropods (with a total number estimated to be in the tens of millions) and the large number of species of plants (270 000) that have been described. It is clear that the anatomical diversity of higher animals and plants provides much more information for classification than the stain-based and biochemical differences among prokaryotes. Curiously, even with our current ability to detect differences in prokaryotes by whole genome comparative analysis, in most cases the

old bacterial 'species' remained as fixed as they were in the pre-genomic era.

Conceptual revision is needed [3–6]. Indeed, the groups of organisms recognized as bacterial 'species' are more consistent with the genus level of classification in eukaryote systematics. As an example, genomic diversity among the mammal species is much smaller than that among different *Escherichia coli* clones. Undoubtedly, if the genomic differences used for mammal speciation were applied to prokaryotes, the diversity of bacterial microorganisms would be much more appreciated. That does not mean that particular bacterial clades would necessarily be extraordinarily diverse; for instance, SAR11, an alpha-proteobacterial clade, might constitute, in some ocean areas, 50% of the total surface microbial community, and is probably among the most successful organisms on earth [7]. What is clear is the importance of identifying and measuring microbial biodiversity in order to understand the roles of bacteria. Microorganisms are critical to basic functions in nature—for human and animal health, for food supply, for biodegradation, for waste water treatment, or for biosynthesis of useful compounds. For example, in nutrient recycling, cyanobacteria constitute up to 70% of the total phytoplankton mass, and are responsible for more than 25% of the total free O<sub>2</sub>, and about an equivalent proportion of CO<sub>2</sub> fixation.

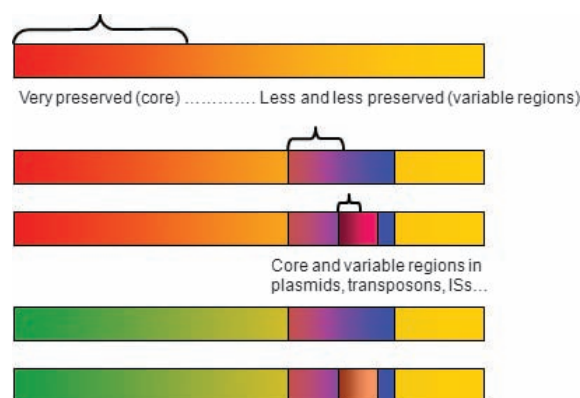
## Exploring diversity beyond species

What we conventionally know as the 'species' should not be considered the ultimate unit for exploration and assessment of diversity in the microbiosphere. Diversity occurs at different levels, many of them sub-specific (e.g. clonal complexes, clones, plasmids, transposons, integrons, genes, and gene modules) and many others supra-specific (e.g. meta-populations, communities, and ecosystems). We obviously need comprehensive methods to build an integrated view of multi-level diversity. One of the main questions in assessing the utility of the concept of evolvability is how to identify what specifically characterizes each one of the sub-specific or supra-specific units, e.g. the 'clonal species,' 'plasmid species,' 'transposon species,' 'integron species,' or 'community species'. To explore this utility, invoking the *law of preservation of cores* is critical.

## The law of preservation of cores

According to its original philosophical meaning, a species is defined by an ensemble of 'substantial' properties that are

highly specific for a particular group of entities and shared by all of them, while the entities might differ in 'accidental' traits. The presence or absence of accidental traits does not influence the assignment of an organism to a particular species. This definition underlies the concept of an immutable core as opposed to a variable, accessory set of structures. This concept of species applies to everything that has constant and variable parts. An interesting property of nature is its fractal nature; that is, organizations are repeated with an identical scheme among different hierarchical levels. A bacterial species is defined by bacterial organisms sharing a constant part (the core genome) but differing by accidental traits (the accessory or dispensable genome). The core is, at any hierarchical level, a set of genes sharing a common history [8]. But if we go below this hierarchical level, even into accidental structures, we can find a constant part and a variable part. There are constant and accessory parts in plasmids, and, in the accessory parts of plasmids (transposons), we can also find core and accessory parts. Integrons, which might be considered as accessory parts of transposons, also have core and accessory sequences (Fig. 1), and the same is true for genes and protein sequences. The logic of the system is that at any hierarchical



**Fig. 1.** The law of preservation of cores. Bars correspond to particular biological genetic elements. In all bars, including the medium-sized and small ones inserted in the larger ones, there is a gradient of variability; in the top bar, the part in which the red dominates is the more preserved part (core). In the second long bar, a medium-sized bar has been inserted in the variable region; this bar also has a gradient of variability, being the red-violet part more preserved (core) than the blue part. In the third long bar (from the top) a new small bar has been inserted in the middle-sized bar of the preceding image; this small bar also has a core region (dark violet) and a variable region. The last two long bars illustrates the possibility that the same elements (medium-sized) or different elements (small ones) could be inserted into big bars. Note that at any hierarchical level (hierarchies corresponds to the bar's size) there is a core (shown approximately in brackets) and variable regions.

level, a core is always preserved and, to preserve the core, the accessory parts are needed. This law of preservation of cores explains the asymmetry among the relatively frequent micro-evolutionary events (evolutionary changes occurring among the accessory structures) and the rare macro-evolutionary events (evolutionary changes occurring in the cores).

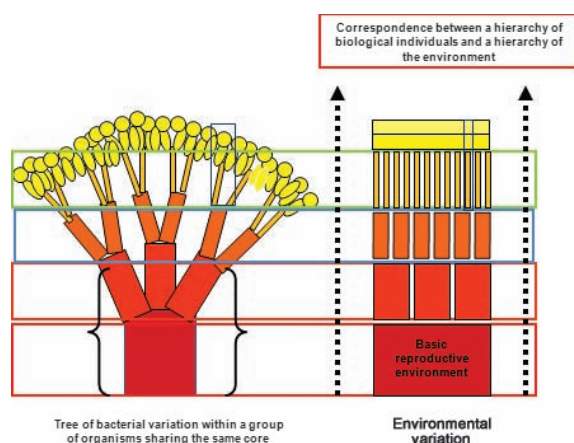
### Correspondence between the biological hierarchy and the hierarchy of the environment

The existence of cores is explained by the necessity of preserving integrated networks of functions that ensure the fitness of the structure that harbours the core in a particular environment. That we find associated accessory structures linked to cores should reflect increased fitness in sub-environments. We do not suggest that all information contained in any accessory sequence necessarily provides an adaptive advantage. Nevertheless, most accessory elements contain information of adaptive advantage, or are involved in the spread of such information by lateral genetic transfer. The presence of apparently neutral sequences probably reflects the historical physiological background of such elements (the founder effect), or the remains of old, adaptive functions. In any case, the genetic fixation of the traits involved in each gain in an adaptive function should be explained by the increased fitness in the corresponding environment(s). Because of that, we could envisage the concept of a 'hierarchy of environments'

that mirrors the hierarchy in biological variation. To a certain extent, there should be an 'environmental species' corresponding to each 'biological species' [9], and the same might be applied to biological subspecies or clones. The notion of ecotypes, groups playing ecologically distinct roles, or the concept of 'envirome', i.e. the virtual environment as it can be deduced from the presumed ecological functions of the genome, illustrates such correspondence [4,10]. These concepts are critical to an understanding of the potential effects of environmental damage on the microbiosphere. Changing an environment means the modification of one of these 'environmental units of selection' that influences the persistence of a particular biological variant. As these changes necessarily have 'up-the-hierarchy' and 'down-the-hierarchy' effects, even minor changes in the environment might influence changes in the genetic composition of a microbial system. The correspondence between biological hierarchies and environmental hierarchies is illustrated in Fig. 2.

### Correspondence of genetic variation and environmental variation

The recent increase in knowledge of the population biology of microorganisms, resulting from the wide application of techniques such as whole genomic sequencing, or multilocus sequence typing and pulsed-field gel electrophoresis typing, has revealed the hierarchical structure of clonal variation within bacterial species. In many cases, there is a high diversity of variants (e.g. sequence types in multilocus sequence typing), but many of them are clustered around a putative founder clone of further phylogenetic lineages (clonal complexes). A number of clonal complexes are, in turn, clustered together in higher phylogenetic 'groups'. In many cases (as with *E. coli*, *Enterococcus faecium*, or *Enterococcus faecalis*), such groups are composed of strains that originated in particular environments (e.g. the intestinal tract of a given mammal) [11]. Such environments are the basic reproductive environments for such strains. Second-order environments could explain the existence of clonal complexes, or third-order environments the bacterial diversity at lower levels. But even within a particular environment, variants may occur, as the environment might change in time. Therefore, different clones peak in frequency at different times, according to the which clone fits best in each epoch of a changing environment. The maintenance of clonal ensembles is favoured by the asymmetry of fitness abilities in different clones in different epochs. The metastability of the species at large is ensured by its internal clonal diversity (eventually maintained by internal competition) and intermittent clonal



**Fig. 2.** On the left, a symbolic image of a bacterial species, sharing a common core (down), that is being diversified to form individual clones (or bacterial ecotypes). The environment in which such a species is located (right part) has a similar structure; the basic environmental features correspond to the core (basic reproductive environment); there is a hierarchy of 'environment ecotypes' corresponding to each bacterial ecotype.

fixation. The same is true at every level of the hierarchy; there is a metastability of 'plasmid clones,' 'transposon clones,' or 'integron clones,' each of them sharing a common 'core' [12,13].

### **A hierarchical evolutionary structure: chinese boxes**

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Owing to the intrinsic complexity of biological systems, the requirements for adaptive changes should be compatible with the basic interactive networks governing the physiological mechanisms of the cell in particular environments. The word 'mechanism' recognizes a mechanical interplay between pieces and, in this sense, evolution should be an engineering process [3]. Modularization is the basic evolutionary tool that makes the required adaptive variation compatible with preservation of the physiological network. Modularization works by the insertion or replacement of pieces in genetic regions, thereby ensuring minimal disturbances in the host's physiology [14]. These regions tend to become multimodular, and the relative neutrality of such complexes makes them suitable for lateral genetic transfer. Indeed, this phenomenon is particularly visible in bacterial plasmids and transposons. On the other hand, such modular engineering occurs across all hierarchical levels, creating specific 'Chinese box' or 'Russian doll' patterns of stable (preferential) combinations of bacterial species, phylogenetic sub-specific groups, clones, plasmids, transposons, insertion sequences, and genes encoding adaptive traits, such as antibiotic resistance. Assuming a relatively high frequency of combining events, trans-hierarchical combinations probably result from the local availability of the different elements (pieces) in particular locations (local biology), the historical and local advantage provided by particular combinations, and the biological cost in fitness of each combination. More research is needed to understand the interactive pattern of biological pieces in particular environments (grammar of affinities). It could be useful to have in mind a possible 'return to chemistry' to understand our evolutionary scenario. The central Michaelis-Menten equation, used to predict the interactions between an enzyme and a substrate, can perhaps be applied here, at least as a metaphor. It could allow predicting  $V_{max}$ , the approximate speed of evolutionary interaction, by considering  $S$ , the approximate local concentration of evolutionary pieces influencing connectivity, and  $K_m$ , the approximate affinity (including historical linkages) of evolutionary pieces. Knowledge of such types of availabilities and affinities for genetic elements might enable us in the future to establish 'chemical-like' predictions to help understand the evolutionary trajectories of microorganisms and microbial systems [15].

### **Trans-hierarchical cooperation and individuality**

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The genetic structures of replicating entities, at all hierarchical levels, benefit greatly from useful functions provided by genes. A gene providing a useful function immediately increases the possibility of survival of the platform in which it is located. Indeed, the platform might include several advantageous genes, further increasing its biological net value. If such a platform is acquired by an even higher hierarchical structure, the higher structure will increase its own biological value. Also, every benefit for the higher structure means a return benefit for the lower hierarchical structures. At all levels, the fate of every hierarchical unit depends on the other hierarchical units, higher or lower. Because of this, trans-hierarchical cooperation tends to be preserved.

A problem arises when a hierarchical unit that contains a useful (cooperative) sub-hierarchical structure acquires another structure that potentially reduces the fitness of the pre-existing one. In that case, the only possibilities are: (i) creation of a novel structure by recombination, with a certain risk of incompatibility with other higher or lower hierarchies, but also with potential benefits; (ii) modification of the higher or lower structures to fit with the new incoming structure; and (iii) rejection of the new incoming structure. At certain levels of maintained and refined hierarchical cooperation, rejection is the best way out. When any hierarchical structure rejects new incoming elements, it becomes an individual. Therefore, an individual is a cohesive whole, a closed system of trans-hierarchical interactions, and its evolutionary outcomes depend on multilevel selective events.

### **Expanding the concept of inclusive fitness to trans-hierarchical individuals**

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William D. Hamilton proposed in 1964 the concept of inclusive fitness, referring to the sum of reproductive success of all individuals in a community, plus the effects that each individual has on the reproductive activity of its genetic relations. It becomes clear from the discussions above that the notion of reproductive fitness applies not only to the individual bacterial cells, or the clones, but also to any other replicating unit, including subcellular replicons such as plasmids or transposons. As noted, increases or decreases in fitness of subcellular replicons should influence cellular and population fitness. In such a perspective, the Hamiltonian view of inclusive fitness could be applied to trans-hierarchical

relationships. Mathematical methods could be used to explore this, but we stress that environmental changes affect the fitness of complex trans-hierarchical individuals. In a sense, these trans-hierarchical individuals constitute systems, and environmental changes necessarily have systemic consequences.

### Variation is both reduced and increased by environmental stress

Environmental stress is produced when a change in the environment reaches or surpasses the limits of physiological tolerance of the biological unit exposed to it, at any level of the hierarchy. Stress is transmitted along the hierarchy; for instance, an environmental change that reduces the fitness of a particular species might contribute to the selection of one of the composing clones, and as particular plasmids might be preferentially linked to a given clone, the plasmid population will be non-optimally adapted to the selected clone; that is, the plasmid also reduces its fitness. These cases illustrate two of the key features of environmental stress considering as a consequence of its selective effect for a particular type of genetic variant, comprising those associated with a higher fitness in the stressful situation. The first is that environmental stresses *reduce* population's of genetic variability. The second is that sub-selective and possibly peri-selective environmental stresses *increase* variability.

But environmental stress is generally heterogeneous in space; in some areas it will be very high, and its strength will progressively decrease with distance from the centre. That means that a central zone of reduction in variation will be surrounded by a zone of increased variation, from which mutants better able to cope with environmental stress might eventually arise. In general, gradients of stressful conditions should facilitate an increase in genetic diversity [16–18]. Of course, random environmental changes give rise to gradients in which a certain pressure to maintain a basic level of diversity occurs [19].

A number of observations illustrate the induction of genetic variability by stress. Mutation can be considered a stress response, and the evidence for the existence of various stress-induced mutagenesis programmes is now overwhelming [20]. In *E. coli*, there are polymorphic mutation frequencies that apparently relate to environmental stability. Strains from the intestinal content of healthy people have reduced mutation rates in comparison with invasive strains isolated from blood, or with strains that are multiresistant to antibiotics and that have been previously exposed to antibiotic stress [21–23]. *Pseudomonas aeruginosa* strains from chronic infections, sub-

jected to frequent antibiotic and immunological exposure, are hypermutagenic in comparison to those isolated from acute infections [24]. Horizontal gene transfer occurs more frequently under stressful conditions [25–27], and such transfer accelerates genome innovation and variation [28,29]. Recombination occurs more frequently during stress, sensed or not through the SOS system [29–31].

### Evolution of human–bacteria relationships in a changing environment

Martinez and Baquero have recently summarized [32] 12 major anthropogenic factors that change the environment and that might shape future interactions between humans and pathogenic bacteria. I reiterate them here, with small modifications: (i) sanitation and hygienic measures, reducing access of bacteria (both pathogenic and commensal) to humans; (ii) chemical pollution—including drugs—altering bacterial biodiversity; (iii) human demographic changes, including increased mobility, e.g. increasing host contact with different bacteria; (iv) global environmental changes, leading, for instance, to changes in geographical localization of pathogens; (v) increase in the number of hypersusceptible hosts for infections; (vi) new medical technologies, opening new opportunities for the development of opportunistic organisms; (vii) intensive farming, reducing animal biodiversity and therefore microbial diversity; (viii) expansion of viral diseases, eventually altering host susceptibility; (ix) new antibiotic and antimicrobial strategies; (x) vaccination and immunomodulation; (xi) bioterrorism and biological warfare; and (xii) science, promoting awareness of these effects and, possibly, supplying solutions. An important part of these solutions should be the continuous monitoring of the diversity of the evolutionary units, at any hierarchical level, that shape microbial systems. Systematic comparison of diversity changes with environmental changes could lead to the possibility of fulfilling one of the fundamental tasks of biological sciences—the prediction of evolutionary trajectories [15].

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## Transparency Declaration

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# The evolutionary history of symbiotic associations among bacteria and their animal hosts: a model

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## Abstract

A model to explain the evolutionary history of animal-bacteria obligatory mutualistic symbiosis is presented. Dispensability of genes and genetic isolation are key factors in the reduction process of these bacterial genomes. Major steps in such genome reductive evolution, leading towards primary endosymbiosis, and the possibility of complementation or replacement by a secondary symbiont are also indicated. Yet, we need to understand what happens at the beginning of the adaptative process towards an obligate mutualistic relationship. For this purpose, we propose to sequence the complete genome of SOPE, the primary endosymbiont of the rice weevil.

**Keywords:** Complementation, endosymbiosis, genome reduction, replacement, SOPE (*Sitophilus oryzae* primary endorsement)

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## The establishment of symbiosis among bacteria and their animal hosts

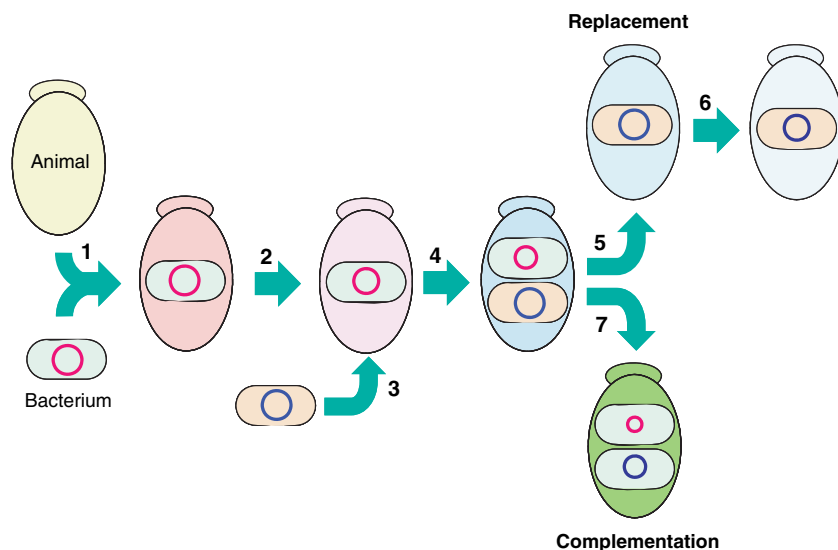
Many bacteria maintain symbiotic associations with eukaryotic cells; this association can be mutualistic, commensal, or parasitic. In some cases, the relationship is so close that the bacteria live inside the host cell, an association called endosymbiosis.

Bacterial endosymbioses are widespread among higher life forms. Insects have been particularly well studied, where the establishment of symbiotic associations with bacteria has allowed them to grow on imbalanced food resources (e.g. plant sap, cereals, or blood), poor in essential nutrients but with the nutrients being provided by the bacteria [1,2]. The symbiotic bacteria reside in specialized host cells called bacteriocytes, and are vertically transmitted from the mother to the offspring. Transition to an obligate intracellular lifestyle in bacteria triggers a cascade of changes that shape the genome structure and content, leading to a reduction in genome size and an increase in the A + T content, among other features. Fig. 1 shows a model for the establishment and evolution of symbiotic associations. The first step

towards the establishment of an obligate endosymbiosis occurs when a free-living bacterium infects a host. From this point, both organisms co-evolve to adapt to the new situation. The host develops specialized cells to harbour the bacterium, which, in turn, provides essential nutrients. In this new stable situation, the bacterium suffers an evolutionary genome reductive process (see next section). Eventually, a second bacterial species might join the consortium. Although, initially, this new association might be facultative, if the second bacterium provides benefits to the association, a new stable association can appear and both bacteria will subsequently co-evolve. As the reductive process continues, and new genes are rendered unnecessary owing to redundancy, two possible outcomes can occur. Either both bacteria will be needed to keep a healthy consortium (complementation) or, depending upon which genome is affected by the loss of genes needed for the synthesis of molecules essential for the association, one bacterium can begin an extreme degenerative process, which may end with its extinction; in this case, the retained bacterium will continue the reductive process alone (replacement).

## Factors affecting the process of genome reduction

As a consequence of their access to an intracellular environment, free-living bacteria alter their genome composition, sometimes in a dramatic way. Within a protected and stable environment, many genes are rendered unnecessary, whereas others become redundant because their functions



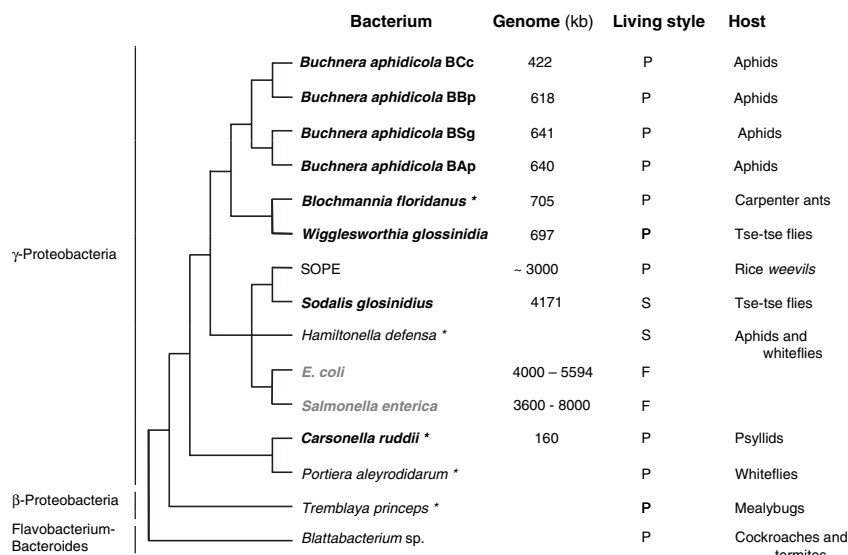
**Fig. 1.** Major steps in the evolution of endosymbiosis. See text for more details.

can be supplied by the host. As a consequence, genetic material can be lost without a detrimental effect and can accumulate mutations because of the lack of natural selection to purge them. In addition, as compared to the high numbers of free-living bacteria in adult hosts, relatively few endosymbionts are maternally inherited by the offspring of the host. Thus, such systematic bottlenecking over generations favours the action of random drift [3]. Another consequence of living in an intracellular environment is that bacterial endosymbionts remain relatively isolated, which favours the lack of horizontal gene transfer and DNA intake [4]. To summarize, dispensability of highly mutated and non-essential genes, and genetic isolation, are key features in the process of genome reduction.

## Empirical evidence from insect endosymbiosis

Fig. 2 shows most of the bacterial insect endosymbionts that have been analysed, the majority of them belonging to the  $\gamma$ -proteobacteria. They can be regarded as primary endosymbionts (essential for its host fitness and survival) or secondary endosymbionts (when they are facultative or can live outside of the eukaryotic cells).

We are interested in studying the evolution of these genomes to identify which changes occur during their adaptation to intracellular life and the processes that cause them, as well as to determine factors involved in establishing a



**Fig. 2.** Bacterial symbioses among insects. Phylogenetic relationships among the main bacterial symbionts of insects that have been analysed are shown. Species whose genomes have been completely sequenced appear in bold. Free-living relatives appear in grey. P, primary endosymbiont; S, secondary symbiont; F, free-living. \*The species name is under the category *Candidatus*.



mutualistic relationship instead of a pathogenic one. The sequencing of genomes from bacteria with young and old endosymbiotic relationships with their eukaryotic hosts will assist in that task. All the primary endosymbionts analysed so far have genome sizes about eight to ten times smaller than their free-living relatives. One of the most extreme cases, that of *Buchnera aphidicola* from the aphid *Cinara cedri* (BCc strain), has recently been sequenced in our laboratory and might represent a final stage in the endosymbiotic relationship [5]. In all previous cases analysed, *B. aphidicola* provides essential amino acids plus riboflavin to the aphid host. However, *B. aphidicola* BCc has lost the ability to synthesize tryptophan and riboflavin, and most retained genes are evolving at a faster rate [5]. In addition, another endosymbiont, *Serratia symbiotica*, has been found in all analysed cedar aphid populations. On the basis of all these findings, we postulate that *B. aphidicola* BCc is losing its symbiotic capacity and is being complemented (and might be replaced) by the highly abundant coexisting *S. symbiotica*.

To better understand the molecular and evolutionary basis of symbiosis, it is necessary to sequence and characterize the genome of a bacterium in the first stages of the process. For this purpose, we chose SOPE, the primary endosymbiont of the rice weevil, *Sitophilus oryzae*. SOPE is a  $\gamma$ -proteobacterium that maintains a typical obligate mutualistic endosymbiosis with its host. The bacteria live inside bacteriocytes organized in an organ, called a bacteriome, surrounding the midgut of the insect and near the female ovaries. The bacterium cannot be cultured outside the host, and it provides to the insect at least amino acids and vitamins, which have effects on fertility, development, and the flying ability of adult insects. The age of this symbiosis has not been completely established, but there are two key factors that indicate that it must be a young relationship: its relatively large genome size (3 Mb, as estimated by pulsed-field gel electrophoresis), and its G + C content, which is high in comparison to that in genomes from other insect endosymbionts but similar to that found in free-living proteobacteria [6,7]. SOPE may be a replacement of an ancestral endosymbiont in the family Dryophthoridae, to which the rice weevil belongs [8]. SOPE is closely related to *Sodalis glossinidius* [9,10], a secondary symbiont found in the tsetse fly, whose genome has recently been sequenced [11]. In contrast to SOPE, *Sodalis* can be cultured in the laboratory and is found intracellularly and extracellularly in its host. Comparing the

genomes of these two bacteria will allow us to determine which genes are lost at the beginning of the adaptive process, and to draw some conclusions about the process of adaptation of bacteria to endosymbiotic life.

## Transparency Declaration

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# Towards an ecological approach to antibiotics and antibiotic resistance genes

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## Abstract

Antibiotics are likely the most important compounds used for human therapy. Conversely, antibiotic resistance is a relevant medical problem. However, besides their relevance for human health, antibiotics and their resistance genes are important elements that can influence the structure of microbial populations. In this article, we discuss antibiotics and antibiotic resistance genes in non-clinical environments.

**Keywords:** Antibiotic resistance, bacterial evolution, microbial ecology, subinhibitory concentration

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Antibiotics (including synthetic antimicrobials) are probably the most successful therapeutic agents developed by humans. Although sulphonamides, the first widely used antibiotics, are synthetic, the large majority of these compounds have a natural origin. Furthermore, production of antibiotics is a frequent characteristic of environmental microorganisms. Conversely, antibiotic resistance is widely disseminated in non-clinical environments, and antibiotic producers contain a large number of antibiotic resistance genes in their genomes [1] that might eventually be transferred to pathogenic bacteria.

Antibiotic producers are able to outcompete, at least under laboratory growing conditions, other microorganisms [2]. Thus, a suitable ecological role for these molecules in nature could be the Darwinian struggle for life against competitors. As Waksman and Woodruff stated in 1940, the reason for searching for antibiotic producers in soil was the assumption that this type of organism must be present in the environment and that production of antimicrobials should have an ecological role in inhibiting the growth of other microorganisms: 'The soil was searched for bacterial agents of infectious diseases, until the conclusion was reached that these do not survive long in the soil. It was suggested that the cause of the disappearance of these disease-producing organisms in the soil is to be looked for among the soil-

inhabiting microbes, antagonistic to the pathogens and bringing about their rapid destruction in the soil' [3].

Following the same reasoning, the presence of large numbers of antibiotic resistance genes in natural (non-clinical) environments strongly suggests that they must have an ecological role, either in endogenous detoxification in the case of producer organisms, or in protecting bacteria from the action of exogenous antimicrobials.

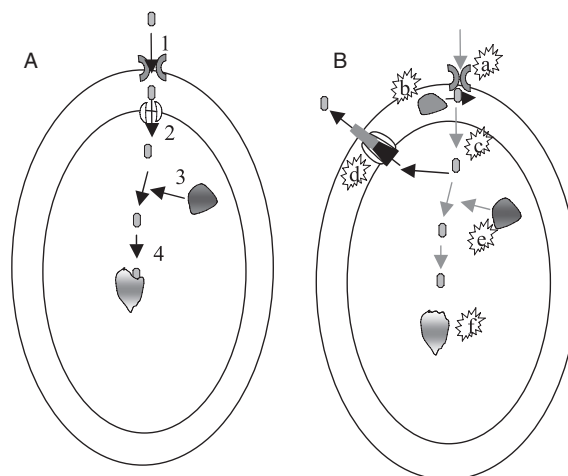
This 'weapon/shield' function for antibiotics and their resistance genes is useful for pathogenic bacteria growing in a treated host. This is an ecosystem with high concentrations of antibiotics, where the unique function of these molecules is to inhibit bacterial growth. Being resistant in an environment with a high load of antibiotics is a rewarding strategy because it allows the utilization of resources in the absence of competitors, which are being killed by the antimicrobial. Antibiotic resistance can be considered as a colonization factor in the presence of antibiotics [4].

Do antibiotics and antibiotic resistance genes play the same role in non-clinical environments? This may be true in some instances, but compelling evidence indicates they may have very different roles in mediating the interactions of microbial communities in natural environments. There are two important key concepts. First, the toxicity of a molecule is always concentration-dependent. Furthermore, multiple compounds are beneficial at low concentrations and toxic at high ones, an effect that has been called hormesis [5]. For example, iron is a cofactor in several biomolecules and is required for life. However, at high concentrations, iron is toxic. For this reason, all organisms have developed complex and exquisitely regulated mechanisms for acquiring iron and avoiding its toxic effects. Second, production of antibiotics

(and, thus, their concentrations in natural, non-clinical environments) is highly regulated in the producer organisms (a situation that obviously does not occur during clinical treatment of infection). Similarly, the expression of antibiotic resistance genes is finely tuned in the organisms in which they evolve, whereas this regulation does not occur after horizontal gene transfer to a pathogenic bacterium.

The concentrations of antibiotics in natural environments is frequently too low to play their proposed functional role as inhibitors of competitors. Antibiotics are frequently produced at stationary growth phase, not during active growth when the production of a weapon to inhibit competitors would be a rewarding ecological strategy. As antibiotics are usually present at low concentrations in natural environments, studies on the effect of antibiotics at subinhibitory concentrations may give insights into the role that these molecules play in natural ecosystems [6]. These studies have demonstrated that each antibiotic produces a rather specific transcription pattern in global bacterial gene expression [7]. Functional analyses have also demonstrated that antibiotics can trigger expression of phenotypes, such as biofilm production or cytotoxicity, that can give an ecological advantage to bacteria growing under low antibiotic concentrations [7,8]. From these studies, it has been suggested that antibiotics may be signalling molecules mediating intercellular communication in natural environments [8]. The more classic role of 'weapon', with production of high concentrations of antibiotic, might occur in some local micro-environments in the rhizosphere.

If antibiotics are not always weapons, it is possible that the natural function of some antibiotic resistance genes is also other than to encode shields. Bacteria acquire resistance by a few mechanisms (Fig. 1), some of them as the consequence of mutations [9], and some due to horizontal gene transfer [10]. The most frequent antibiotic resistance genes acquired by horizontal gene transfer are antibiotic-inactivating enzymes and efflux pumps. It was earlier stated that aminoglycoside-inactivating enzymes could have a metabolic role, in addition to a function in resistance, because 'phosphorylated streptomycin might be important as a metabolic precursor' [11]. More recent work has shown that some aminoglycoside-modifying enzymes may be involved in acetylation of peptidoglycan [12]. The similar structure of the enzymatic substrate and the antibiotic would allow these enzymes to confer resistance even though they evolved to play a completely different role. The investigation of sequenced microbial genomes indicates that this could be a frequent situation, because all bacteria contain in their genome antibiotic resistance genes, whether the bacteria produce antibiotics or not. As these genes are present in all



**Fig. 1.** Mechanisms of antibiotic resistance: (A) A schematic representation of a Gram-negative bacterium with two membranes. To inhibit microbial growth, an antibiotic needs to cross the cellular envelopes (1 and 2), on some occasions to be modified (3) and to reach its target (4) at concentrations high enough for its effective binding. (B) Mutations in all the elements involved in this pathway can produce resistance because the membrane transporters suffer structural changes (a) or are not expressed (c), because there is a modification (e), or lack of expression of, the enzyme that activates the pre-antibiotic or because the target presents structural modifications (f) that impede the antibiotic's binding. Antibiotic-inactivating enzymes (b) and efflux pumps (d), either present in the bacterial genomes or acquired by horizontal gene transfer, also contribute greatly to antibiotic resistance.

members of each species, they have not been acquired recently by horizontal gene transfer as the consequence of antibiotic pressure from antibiotics used by man and probably have functional roles different to resistance.

Multidrug resistance efflux pumps constitute another category of antibiotic resistance genes for which a functional role other than resistance has been proposed. Common substrates of efflux pumps are the quinolones, and it has been demonstrated that environmental bacteria isolated before the discovery of these synthetic drugs were capable of extruding quinolones [13]. Notably, some multidrug pumps from *Pseudomonas aeruginosa* can extrude signal molecules involved in quorum sensing in this bacterial species, including one natural quinolone-like compound. Overexpression of those pumps increases resistance to several antibiotics and simultaneously leads to a reduction in the quorum sensing response and virulence [14–16]. It is thus likely that these efflux pumps might be involved in the bacterial response to intercellular signalling.

In summary, we propose that antibiotics may be signalling molecules (and not only weapons) in natural environments.

Conversely, antibiotic resistance genes could have metabolic roles (and act not only as shields) that may include biosynthesis of macromolecules, maintenance of homeostasis, and signal trafficking.

## Transparency Declaration

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## Fungi sensing environmental stress

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### Abstract

Cells need to adapt to the external environment in order to survive. Signal transduction pathways are crucial mechanisms that allow cells to sense and respond to extracellular stimuli. Among the signal transduction pathways, we point out the cascades mediated by mitogen-activated protein kinases (MAPKs). The MAPKs are conserved from yeast to human and play relevant roles in the physiology of the cell. In pathogenic fungi these MAPK pathways control virulence factors. This review describes the MAPK cascades described in *Candida albicans*, the most frequently isolated fungus, from fungal systemic infections among individuals in developed countries.

**Keywords:** *Candida albicans*, fungi, mitogen-activated protein kinase, signal transduction

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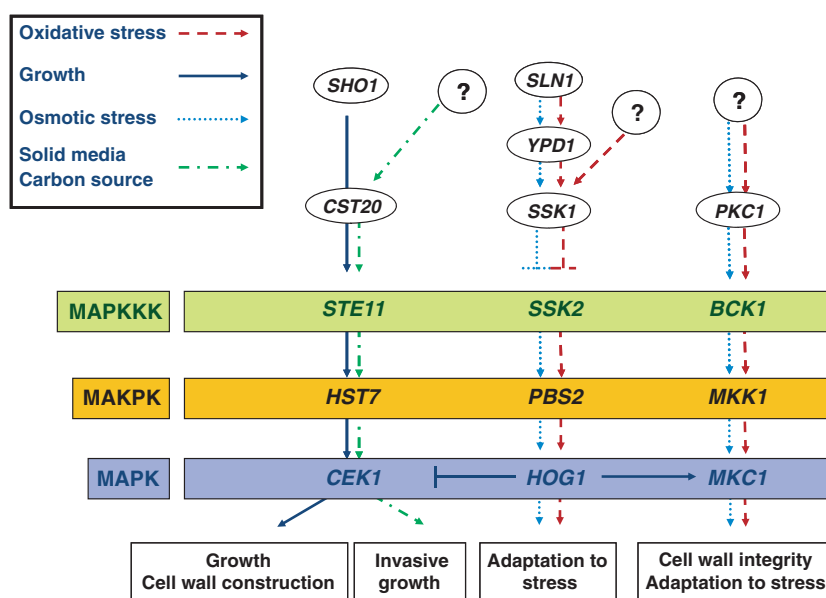
Eukaryotic cells respond and adapt to external stimuli mainly through signal transduction pathways mediated by mitogen-activated protein kinases (MAPKs). These pathways are well conserved, from the simplest to the most complex organisms, and maintain a similar structure [1]. There is a central MAPK core formed by three MAPKs (MAPK, MAPK kinase and MAPK kinase kinase) that sequentially activate each other by phosphorylation. The signals are sensed by specific receptors that trigger the central module directly or through intermediate proteins. The final effectors of the cascade are, mainly, transcription factors that adjust the transcriptional response, allowing adaptation to environmental change.

Responses to external stresses or vegetative growth are among the processes regulated by MAPK pathways. Therefore, MAPKs are central to a network of pathways that integrate, amplify and modulate protective and adaptive responses. Remarkably, MAPK signalling pathways control virulence in pathogenic fungi. In recent decades, the numbers of infections caused by opportunistic fungi have increased enormously, principally in developed countries. This increase is the consequence of factors such as the immunosuppression linked to organ transplants, cancer, chemotherapy, etc., or the increase in numbers and complexity of invasive techniques (parenteral feeding, catheters, etc.). The most frequently isolated fungal species involved in these infections are *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus*

*fumigatus*, but others have also been identified with increasing frequency. *C. albicans* is still the fungus most frequently isolated from systemic infections. This microbe bases part of its successful strategy of colonization of the host on its ability to assume different morphologies, depending on the growth conditions [2]. They range from unicellular forms (yeast-like forms) to hyphae, but pseudohyphae or thick-walled spores (chlamydospores) are also found. *Cryptococcus neoformans* is a basidiomycetous fungal pathogen that causes human infections after inhalation of small, desiccated yeast cells or spores that pass into the alveoli of the lung [3]. It is the most common cause of fungal infections of the central nervous system, causing fatal meningoencephalitis if untreated. Over the past decade, invasive aspergillosis, which is most often caused by *A. fumigatus*, has emerged as the most serious life-threatening infectious complication of intensive remission-induction chemotherapy and allogeneic bone marrow transplants, and in patients with various haematological malignancies [4].

At least five cascades have been described in *Saccharomyces cerevisiae*, and they are involved in mating, adaptation to hyperosmotic conditions, cell wall integrity, invasive or pseudohyphal growth, vegetative growth, and ascospore wall formation. Several orthologues of signal transduction genes present in these non-pathogenic yeasts have been found in pathogenic fungi. In general, the MAPK pathways conserve similar functional structure and organization; nevertheless, important differences exist that reflect the specialization of the cascades and, in turn, also influence their role in virulence.

Four MAPKs have been identified and characterized in *C. albicans*: Mkk1, Hog1, Cek1, and Cek2 (Fig. 1). Remarkably, Mkk1 and Hog1 are involved in the response to oxida-



**Fig. 1.** A scheme of the mitogen-activated protein kinase (MAPK) signalling pathways identified in the pathogenic fungus *Candida albicans*.

tive stress, triggered by phagocytes to fight pathogenic microorganisms. The Hog1-mediated MAPK pathway is involved in resistance to osmotic stress and, interestingly, also to oxidative stress, heavy metals, and thermal shocks [5,6]. In addition, it seems to play an essential role in the regulation of cell wall construction, as shown by the differential behaviour against some antifungals that interfere with its assembly, such as Nikkomycin Z and Congo red [7]. It also plays a role in the dimorphic transition, negatively regulating this process, as Hog1 mutants show an increased ability to filament in sub-inducing media. The MAPK of the HOG pathway, Pbs2 [8], is responsible for the activation of Hog1, and pbs2 mutants display phenotypes that overlap with the phenotypes observed in hog1 mutants, having an enhanced ability to filament and showing cell wall alterations. The transmembrane protein Sho1, first placed on one of the branches of the HOG pathway, mediates the activation of the Cek1 MAPK, which is involved in *C. albicans* growth [9] and in the sensitivity to stress and cell wall biogenesis in this fungal pathogen [10]. Oxidative stress is sensed mainly through Ssk1, which is one member of a two-component system that belongs to the second branch identified in the HOG pathway [11]. The Ssk1 regulator also determines an enhanced killing by human neutrophils [12]. Remarkably, the Hog1 mutant is avirulent in a systemic infection model in the mouse [7], and, moreover, this mutant strain displays reduced viability in the presence of phagocytes [13].

The Mkc1 MAPK, a homologue of *S. cerevisiae* Slt2, the cell integrity pathway MAPK, is essential for growth at elevated temperatures and contributes to the biogenesis of

the cell wall. It is also involved in the dimorphic transition and in the biogenesis of the cell wall, as determined by the increased susceptibility of Mkc1 mutants to cell wall lytic enzymes and alterations of the cell wall surface [14,15]. Recent data indicate that this MAPK is also activated by oxidative and nitrosative stress, under different stress situations (ionic, temperature, and certain antifungals) [16] and by contact with surfaces under specific conditions such as those that occur within fungal cells, to initiate invasive growth on solid surfaces [17]. Mkc1 is also a virulence factor, and mkc1 mutants have reduced virulence in a mouse model of systemic infection [18]. This strain is also more susceptible to nitric oxide [19], in close agreement with previous data and data obtained by our group using *C. albicans* mutants altered in the morphological transition [20].

MAPK cascades control most of the virulence factors characterized in *C. albicans*: cell morphology, superficial antigen (cell wall biogenesis), and response to different stresses, among them oxidative and nitrosative stress. These cascades allow opportunistic pathogens to recognize changes in their environment and take advantage of an impaired immunological system to cause infection. A deeper knowledge of the mechanism and regulation of these MAPK cascades could help in the control of candidiasis as well as in the development of effective vaccines against these severe infections.

## Transparency Declaration

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# Antibiotic resistance genes from the environment: a perspective through newly identified antibiotic resistance mechanisms in the clinical setting

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## Abstract

Soil bacteria may contain antibiotic resistance genes responsible for different mechanisms that permit them to overcome the natural antibiotics present in the environment. This gene pool has been recently named the 'resistome', and its components can be mobilized into the microbial community affecting humans because of the participation of genetic platforms that efficiently facilitate the mobilization and maintenance of these resistance genes. Evidence for this transference has been suggested or demonstrated with newly identified widespread genes in multidrug-resistant bacteria. These resistance genes include those responsible for ribosomal methylases affecting aminoglycosides (*armA*, *rtmB*), methyltransferases affecting linezolid (*cfz*) or plasmid-mediated efflux pumps conferring low-level fluoroquinolone resistance (*qepA*), all of which are associated with antibiotic-producing bacteria. In addition, resistance genes whose ancestors have been identified in environmental isolates that are not recognized as antibiotic producers have also been recently detected. These include the *qnr* and the *bla*<sub>CTX</sub> genes compromising the activity of fluoroquinolones and extended-spectrum cephalosporins, respectively. The application of metagenomic tools and phylogenetic analysis will facilitate future identification of other new resistance genes and their corresponding ancestors in environmental bacteria, and will enable further exploration of the concept of the resistome as being a unique reservoir of antibiotic resistance genes and genetic elements participating in resistance gene transfer.

**Keywords:** Clinical setting, environment, metagenomic, resistance genes, resistome

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resistance determinants and the corresponding surrounding genetic platforms.

## Pathways for resistance

## Introduction

During the last decade, an increase in rates of antimicrobial resistance has been recognized worldwide, and an increased frequency of multidrug-resistant (MDR) isolates in the clinical setting has been demonstrated. Moreover, the term extreme drug resistance has been applied to those isolates for which there are no available therapeutic options [1]. The accelerated emergence and description of new mechanisms of resistance and the recognition of efficient surrounding genes and genetic platforms facilitating capture, transfer and expression of resistance determinants are also subjects of intense investigation. These studies also include the origin of the

To become resistant to antimicrobial agents, bacterial communities use different strategies, including both natural and engineering responses [2]. Natural responses include: (i) the use of pre-existing bacterial machinery, i.e. the use of resistance genes from other bacteria; and (ii) gene variation (mutation) in pre-existing genes (pro-active response) or in acquired genes (post-active response). Some of these strategies require engineering processes involving different genetic platforms that are used for mobilization, acquisition and assembly of foreign resistance genes. In addition to these mechanisms, bacterial growth in biofilms might also enhance resistance to antimicrobial agents (physiological response).

Resistance response using pre-existing machinery from natural antibiotic-producing bacteria is widely recognized in resistance pathogens. Table I illustrates different examples involving



**Table 1. Natural reservoirs of resistance determinants affecting different antimicrobial agents**

Antimicrobial group	Mechanisms	Related natural protein	Natural reservoirs
New resistance genes associated with antibiotic-producing organisms			
Aminoglycosides	Acetylation Phosphorylation 16S ribosomal RNA methylation	Histone acetylases Protein kinases Methylases	<i>Streptomyces</i> <i>Streptomyces</i> , <i>Micromonospora</i>
Tetracyclines	Efflux (mar)	Major facilitator superfamily EF-Tu, EF-G	<i>Streptomyces</i>
Chloramphenicol	Acetylation Efflux (mar)	Acetylases Major facilitator superfamily EF-Tu, EF-G	<i>Streptomyces</i>
Macrolides	Target mutation	50S ribosomal subunit	<i>Streptomyces</i>
New resistance genes associated with non-antibiotic-producing organisms			
Fluoroquinolones	Topoisomerase protection	QnrA-like protein QnrS-like protein	<i>Shewanella algae</i> <i>Vibrio splendidus</i>
$\beta$ -Lactams	Hydrolases	PBP (transpeptidases)	<i>Kluyvera</i> spp.
PBP, penicillin-binding protein.			

classic and new antibiotic resistance mechanisms. Most of these have been associated with *Streptomyces* spp. recovered from soil, and involve inactivating resistance mechanisms, efflux processes or antibiotic target modifications [2]. In the last few years, it has been demonstrated that antibiotic resistance mechanisms can also originate in environmental bacteria that apparently do not produce antibiotics. Most mechanisms are cryptic resistance determinants in the natural bacteria that are efficiently expressed in the new host. This fact is exemplified by the CTX-M extended-spectrum  $\beta$ -lactamases (ESBLs), which constitute one of the most successfully spread resistance mechanisms affecting expanded-spectrum cephalosporins, or by the Qnr-related topoisomerase protective proteins, which compromise fluoroquinolone activity [3,4].

The *bla*<sub>CTX-M</sub> genes have been associated with *Kluyvera* spp., whereas the *qnr* genes have been associated with *Shewanella algae*. Both comprise environmental isolates that have not been recognized as antibiotic producers, but carry a *bla*<sub>CTX-M</sub> gene and a *qnrA* resistance gene, respectively. In addition, these organisms do not show intrinsic resistance to either expanded-spectrum cephalosporins or fluoroquinolones. On the other hand, isolates in the clinical setting harbouring these genes are of clinical significance and are rising to prominence within the MDR isolates [3,5].

### Environmental antibiotic resistome and antibiotic extended resistome

In recent years, metagenomic tools have identified resistance genes in isolates recovered from the environment [6–8]. These genes form part of pre-existing machineries in bacterial isolates capable of producing substances with antimicrobial activity or, as recently recognized, as natural intermicrobial signalling molecules [9]. The term ‘antibiotic resistome’ was proposed for the collection of all antibiotic resistance genes in microorganisms, including those from pathogenic and non-

pathogenic bacteria. This term exploits the concept of a unique reservoir of antibiotic resistance genes. It includes resistance genes in antibiotic producers and precursor genes that, under appropriate selective pressure, evolve to act as resistance elements. Most are cryptic resistance genes that are not naturally expressed in environmental isolates [8].

D’Acosta *et al.* [6] were the first to use the antibiotic resistome concept when constructing a library of up to 480 *Streptomyces* sp. strains recovered from different environmental sources and subsequently screened against 21 antibiotics. Within this library, they identified resistance genes in all strains and resistance mechanisms affecting not only natural but also synthetic antimicrobials. Some of these resistance mechanisms, e.g. inactivation mechanisms affecting daptomycin or telithromycin, two of the newest antimicrobials introduced in therapeutics, have not yet been characterized in clinical isolates.

This metagenomic approach has been expanded and is also now applied to surrounding resistance genes and genetic elements participating in resistance gene transfer, and is termed the ‘antibiotic extended resistome’ [7]. This concept is useful for the exploration of environmental diversity in different ecosystems, including broad settings (e.g. healthcare settings of nosocomial transmission) or specific compartments, even in a single patient (e.g. the intestinal compartment), and could be useful for prediction of the future evolution of antibiotic resistance [10]. The analysis of some of the recent antibiotic resistance mechanisms described in the clinical setting reinforces these approaches.

### New resistance genes associated with antibiotic-producing organisms

Aminoglycoside-modifying enzymes, efflux-based mechanisms affecting tetracyclines, acetylases modifying chloramphenicol and RNA methylases conferring resistance to macrolides (Table 1), among others, have been traditionally considered

to be resistance mechanisms, the corresponding resistance determinants for which can be found in natural antibiotic-producing bacteria. This list has now been enlarged with the identification of new resistance genes occurring in these bacteria.

### Ribosomal methylases affecting aminoglycosides

Methylation of the 16S rRNA is the most recently described resistance mechanism affecting aminoglycosides [11]. It was first identified in a *Citrobacter freundii* isolate recovered in Poland in 2002, and then in *Pseudomonas aeruginosa* isolates in Japan and in *Klebsiella pneumoniae* isolates in France in 2003. Although few recent reports have been published, it has been also described in other members of the *Enterobacteriaceae*, including *Proteus mirabilis*, *Serratia marcescens*, *Salmonella* spp., *Enterobacter aerogenes*, *Escherichia coli*, *Shigella flexneri*, *Klebsiella oxytoca*, and *Acinetobacter* spp. Most of these isolates were recovered in clinical settings. In the future, we will surely encounter an increasing number of publications depicting the epidemiological situations in which this resistance mechanism has clinical relevance.

The production of the aminoglycoside methylases has been associated with different gene families, including the *armA*, *rmtA*, *rmtB*, *rmtC* and *rmtD* genes. The amino acid and nucleotide alignment demonstrates that these genes have originated from the *Actinomycetales*, including *Streptomyces* and *Micromonospora* spp. [12]. These genes are linked with insertion sequences (ISs) that have shown integration, mobilization and expression functions, such as *ISCR1* (formerly ORF 513) or *ISEcp1*, similar to those encountered with other emerging resistance genes. All these sequences are carried in transposable platforms within conjugative plasmids, allowing potential spread within bacterial populations. Moreover, it is important to note that 16S rRNA methylase genes have been commonly associated with other resistance genes that are currently well dispersed in human clinical and veterinary isolates, such as *bla<sub>BLEE</sub>* (CTX-M, SHV), plasmid *bla<sub>AmpC</sub>*, *bla<sub>SPM-1</sub>*, different aminoglycoside-modifying enzyme genes, and plasmid-mediated quinolone resistance genes (*qepA*). All of these resistance genes allow potential co-selection and maintenance processes in compartments with high antibiotic use [11]. Recent studies in isolates of human and animal origin have shown that some of these genes (e.g. *armA*) are associated with a class I integron platform structure harbouring *IS26*, whereas other genes (e.g. *rmtC*) are associated with complex potential transposons linked to *ISEcp1* [13–15].

### Methyltransferases affecting linezolid

Linezolid is an oxazolidinone compound that is active against Gram-positive isolates, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococcal isolates. It is a synthetic bacteriostatic compound that inhibits protein synthesis by binding at the P-site in the ribosomal 50S subunit. Linezolid-resistant isolates have rarely been described. Resistance is mostly due to mutations in domain V of 23S RNA, mainly described in clinical methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococcal isolates and laboratory mutants of *Streptococcus pneumoniae* [16]. Deletion in genes encoding the L4 riboprotein has also been described in *S. pneumoniae*, and these deletions confer cross-resistance to macrolides, oxazolidinones and chloramphenicol [17]. Moreover, efflux pumps affecting linezolid have been identified in *Mycobacterium tuberculosis* [18].

Recently, a methyltransferase affecting the linezolid ribosome target and caused by the *cfr* gene was recognized in a clinical *Staphylococcus aureus* isolate recovered in Colombia [19]. The Cfr methyltransferase modifies adenosine at position 2503 in 23S rRNA in the large ribosomal subunit. Although linezolid is a synthetic antibiotic, the *cfr* gene is thought to be of natural origin and could be responsible for resistance mechanisms involving protection against natural antibiotics whose site of action overlaps that of linezolid. The Cfr methyltransferase also affects chloramphenicol, lincosamides, pleuromutilins and streptogramin A antibiotics. In this *Staphylococcus aureus* isolate, the *cfr* gene was located in the chromosome linked to the *ermB* gene, which confers resistance to erythromycin through dimethylation of A2058 in 23S rRNA. A chromosomal location of the *ermB/cfr* operon was previously identified in transposable elements within a conjugative plasmid in coagulase-negative staphylococci of animal origin [20]. This demonstrates dissemination of a resistance mechanism of potential environmental origin affecting a synthetic antibiotic, in this case, linezolid.

### Plasmid-mediated quinolone resistance associated with efflux pumps

Traditionally, quinolone resistance mechanisms have been mainly due to target modifications and efflux pumps. Resistance due to target modifications is determined by topoisomerase mutations (*gyrA* mutations in Gram-negative bacteria, and *parC* in Gram-positive bacteria), whereas resistance due to efflux pumps involves different families of

efflux pumps. Both mechanisms are chromosomally mediated and are increasingly recognized in clinical isolates. During the last few years, plasmid-mediated quinolone resistance mechanisms have been unexpectedly found, including: (i) Qnr topoisomerase-protective proteins; (ii) the AAC(6')-Ib-cr quinolone-modifying enzyme; and (iii) the QepA efflux pump [4,5,21,22]. The last of these, due to the *qepA* gene, was first characterized in multiresistant *E. coli* isolates recovered in Japan in 2002, which also harboured other resistance genes such as *bla*<sub>CTX-M-12</sub>, *bla*<sub>TEM-1</sub>, *rmtB*, and *mphA*. The *qepA* gene has also been characterized in Europe (Belgium) in bacterial collections recovered from 2000 to 2005. These isolates, like those recovered in Japan, harboured other resistance genes, such as *bla*<sub>CTX-M-14</sub>, *bla*<sub>TEM-1</sub>, *dfrA17*, *sulI*, *tet(A)*, *catI*, *ant39*, and *rmtB*, giving a multidrug resistance phenotype.

Sequence analysis of the *qepA* gene and alignment with other genes have revealed a potential origin in transport systems (MFS efflux pumps) from environmental isolates, such as *Actinomycetales* (*Nocardia farcinica*, *Streptomyces globisporus*, and *Streptomyces clavuligerus*). Recent studies demonstrated that the *qepA* gene is linked to transposable elements and IncFI plasmids, allowing mobilization among bacterial isolates [21,22]. These results suggest that there will be future appearances of this resistance mechanism.

But, from a phenotypic view, wild-type isolates expressing the *qepA* gene have higher MICs than those of transconjugants, because of the coexistence of other resistance genes affecting quinolones in the former. Although the QepA efflux pumps confer only a slight incremental increase in MIC values, their presence might favour the development of mechanisms expressing even greater resistance [5]. Coexistence of the *qepA* gene with mechanisms conferring higher fluoroquinolone resistance might also favour the fixation of this newly described resistance gene in the bacterial resistome.

### New resistance genes associated with environmental isolates not recognized as antibiotic producing isolates

As noted, two new types of resistance genes, the *qnr* and the *bla*<sub>CTX</sub> determinants affecting fluoroquinolones and extended-spectrum cephalosporins, respectively, exemplify this possibility of new means of antimicrobial resistance appearing among environmental isolates [3,4]. Both of these recently described genes and mechanisms are expanding rapidly within clinical isolates.

### Qnr Topoisomerase protective proteins

The Qnr plasmid-mediated resistance mechanism was identified in 1998 in *K. pneumoniae* isolates producing  $\beta$ -lactamase (FOX-5), which affects extended-spectrum cephalosporins, including methoxy-cephalosporins [23]. The new mechanism confers protection of topoisomerase from fluoroquinolones [4]. Like the *qepA* gene, despite conferring only low-level resistance to fluoroquinolones, it facilitates the selection of isolates with high-level resistance, including those with topoisomerase mutations [24]. There are different families of Qnr proteins, QnrA, QnrB and QnrS, that share variable amino acid similarities. QnrA belongs to the pentapeptide-repeat family, which is defined by a tandem of five specific amino acid repeats within the protein. Some of these proteins, including McbG, a pentapeptide-repeat protein with 19.6% amino acid identity with QnrA, protects against other naturally occurring peptides affecting DNA gyrase, such as microcin B17. Similar proteins have been identified in *Mycobacterium smegmatis* and *M. tuberculosis* [4].

The presence of *qnr* genes has been mainly identified in clinical isolates of *Enterobacteriaceae* with other resistance mechanisms, such as CTX-M ESBL and other newly described  $\beta$ -lactamases (VEB-I and plasmid AmpC-derived enzymes), aminoglycoside-modifying enzymes, or plasmid-mediated efflux resistance mechanisms [4]. The origin of *qnrA*-like genes was determined using a PCR-based strategy on a series of Gram-negative organisms. The reservoir was identified as *Shewanella algae*, an environmental species from marine and fresh water. Four variants of *qnrA* (*qnrA2*–*qnrA5*) were found to be chromosomally mediated in strains of *Shewanella algae* [25]. Moreover, the progenitor of *qnrS* genes was also found in *Vibrio splendidus*, and genes similar to *qnr* with 40–70% amino acid identity were found in the chromosomes of other water-borne species, including *Vibrio vulnificus*, *Vibrio parahaemolyticus* and *Photobacterium profundum* [26]. These findings indicate that gene exchange has occurred between these environmental organisms and *Enterobacteriaceae*, and also support speculation that, under current intensive fluoroquinolone selective pressure, such genes have entered circulation on mobile genetic elements [4].

A recent report has shown an unusual occurrence of the *qnrS2* fluoroquinolone resistance determinant in environmental *Aeromonas* spp. recovered from a river in an urban area in France. The *qnrS2* determinant was harboured in a newly described genetic platform defined as a mobile insertion cassette ('mic'), resembling a transposon within IncU2 plasmids,

which are ubiquitous in a wide range of environments. Both observations increase the possibility of dissemination outside of natural environmental compartments, and also underscore that these bacteria may play a role as a reservoir of the *qnrS* genes in an aquatic environment, as already shown for other resistance genes such as *tet* genes affecting tetracyclines [27].

Despite these findings, *qnr* gene mobilization could have also been performed by the same genetic elements that have mobilized ribosomal methylase genes affecting aminoglycosides or *bla*<sub>CTX-M</sub> genes hydrolysing expanded-spectrum cephalosporins (see below). In fact, the search for the surrounding platform of *qnr* genes facilitated the identification of their genetic linkage with *ISCR1*, which has been associated with all previously mentioned new resistance genes [3,4,11].

### CTX-M ESBLs

The CTX-M ESBLs exemplify one of the most successful resistance mechanisms that have emerged and spread during recent years. This family of  $\beta$ -lactamases was first described 20 years ago, almost simultaneously in Germany and Argentina. It encompasses five different groups of enzymes (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25), all of them displaying potent hydrolytic activity against cefotaxime, although new variants may also effectively hydrolyse ceftazidime [3].

In recent years, a dramatic increase in these  $\beta$ -lactamases has been found worldwide in different compartments, particularly in *E. coli* in the community setting and also in veterinary isolates. Reasons for this increase include its association with ISs participating in gene mobilization, such as *ISEcp1* or *ISCR1* and phage-related sequences, all of them within conjugative plasmids. Although these plasmids are widely disseminated among clinical isolates and are easily transferred, it is important that *bla*<sub>CTX-M</sub> genes and surrounding genes may also be linked to class I integrons allocated within transposable platforms resembling Tn21 subfamilies. These structures can integrate resistance gene cassettes and are associated with resistance to biocides and mercurial compounds that can participate in co-selection processes. Moreover, it is not unusual to find associations of *bla*<sub>CTX-M</sub> genes with those coding for new emerging resistance mechanisms, e.g. 16S RNA methylases (*armA* and *rmtB* genes), Qnr proteins (*qnrA*, *qnrB* or *qnrS*), acetylases affecting fluoroquinolones (*aac(6')-Ib-cr*) and systems pumping out fluoroquinolones (*qepA*); these genes are found in the same genetic platforms.

From an evolutionary point of view, different *Kluyvera* species have been recognized as progenitors of *bla*<sub>CTX-M</sub> genes:

*Kluyvera ascorbata* for the CTX-M-1 and CTX-M-2 groups, and *Kluyvera georgiana* for the CTX-M-8 group. In addition, phylogenetic analysis showed that *bla*<sub>CTX-M</sub> genes have been mobilized from the chromosome of *Kluyvera* spp. approximately ten times more frequently than genes coding for other class A  $\beta$ -lactamases, which has facilitated the spread of the *bla*<sub>CTX-M</sub> genes. Moreover, the *bla*<sub>CTX-M</sub> genes are descended from a common ancestor that was incorporated in ancient times into the chromosome of the ancestor of *Kluyvera* spp. through horizontal transfer [28]. The expression of *bla*<sub>CTX-M</sub> in *Kluyvera* is modest, as isolates belonging to this genus are normally susceptible to expanded-spectrum cephalosporins. This is not the case in *Enterobacteriaceae*.

The importance of *Kluyvera* spp. in nature in recruiting resistance genes has not been sufficiently investigated. It can also act as a donor of resistance genes to other bacteria. In fact, the ability of *ISEcp1* to mobilize the chromosomal *bla*<sub>CTX-M</sub> gene from one of these *Kluyvera* progenitors was shown in an *in vitro* experiment, and its potential role for ensemble in conjugative plasmids was also demonstrated [29].

### Conclusions

The ancestors of most of the new resistance genes found in MDR clinical isolates have been identified both in antibiotic-producing bacteria and in environmental isolates not recognized as antibiotic producers. Mobilization of these genes from soil bacteria might have occurred by the use of similar genetic strategies, with final insertion in genetic platforms enabling efficient transfer to other organisms. These platforms might recruit multiple resistance genes. The wide use of antimicrobials might determine co-selection processes that ensure persistence of these genes in bacterial communities. The application of metagenomic tools and phylogenetic analysis will not only facilitate future identification of other new resistance genes and their corresponding ancestors in environmental bacteria, but will also exploit the concept of the resistome as a unique reservoir of antibiotic resistance genes and genetic elements participating in resistance gene transfer.

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## Transparency Declaration

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# Human migration and infectious diseases

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## Abstract

Emerging infectious diseases (EID) are defined as diseases that have appeared recently or that have recently increased in their frequency, geographical distribution or both. Commercial globalisation, population movements and environmental changes are the main factors favouring the international spread of microorganisms. Transport and communication development constitutes also a remarkable factor in the worldwide dispersion of microorganisms. The mass movement of large numbers of people creates new opportunities for the spread and establishment of common or novel infectious diseases. A surveillance system to detect emergent and re-emergent infections, a rapid responsiveness of healthcare systems and laboratories, vector control, and the provision of healthcare education programmes to inform the population of how to avoid infections are needed in order to stop the spread of infectious diseases.

**Keywords:** Dissemination, emerging infectious diseases, human migration

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Emerging infectious diseases (EIDs) are defined as diseases that have appeared recently or that have recently increased in frequency, geographical distribution or both [1]. EIDs can be caused by: (i) microorganisms that are unrecognized (e.g. coronavirus, [2]); (ii) microorganisms whose geographical range is increasing (e.g. West Nile virus); (iii) microorganisms whose incidence has increased (e.g. human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis*); (iv) microorganisms whose virulence has changed (e.g. *Neisseria meningitidis*); or (v) microorganisms that have acquired antimicrobial resistance (e.g. *Mycobacterium* spp. and *Salmonella* spp.). New strains of microorganisms that cause known diseases but that have acquired new immunological and virulence characteristics, or that have newly acquired resistance against antimicrobial agents, are frequent causes of EIDs. Infectious diseases caused 15 million deaths in 2002, with respiratory infections (3.9 million), AIDS (2.9 million), diarrhoeal illnesses (1.9 million), tuberculosis (1.6 million) and malaria (1.1 million) being the main causes (<http://www.who.int>); many of these had EID components.

Increasing geographical distribution of microorganisms is a hallmark of EIDs, and commercial globalization, population movements and environmental changes are the main factors

favouring the international spread of microorganisms. Globalization has resulted in the increased movement of people, animals, and food products [3]. Changes in global commerce of livestock and food, including marketing, freightage and storage techniques, have increased the numbers of outbreaks of infectious diseases [4].

Ships represent a mechanism of microorganism dissemination. Most ships use water as a ballast system, and, for example, the ports of the USA receive about 79 millions tons of ballast water every year. In 1990, *Vibrio cholerae* O1, Inaba, biotype El Tor, originating in India was isolated in ballast water from five ships in the ports of Mexican Bay. In addition, cruise ships can disseminate infectious diseases such as diarrhoea, legionellosis (through hot tubs), or influenza.

The development of transport and communication constitutes a remarkable factor in the worldwide dispersion of microorganisms. Ships, aeroplanes or other vehicles can disseminate vectors of microorganisms, such as birds and insects. When insects arrive in a new environment, they have to adapt to the new ecosystem and establish themselves—dissemination to adjacent areas may then follow. An example of this situation is the West Nile virus, which was first identified in 1990 in blood collected from a woman from Uganda. This virus is a common pathogen in tropical Africa, the Middle East, and Eurasia. The first outbreak in western countries occurred in New York in 1999 and affected 59 persons. West Nile virus is disseminated by mosquitoes belonging to the *Culex* genus and by birds. Its propagation has a seasonal component, because mosquitoes are quiescent in winter. Recently, an outbreak was reported in

Italy [5]. Chikungunya fever is another example of the contribution of commerce and tourism to the dispersion of vectors of infectious diseases. This infection is caused by the CHIK virus, a zoonotic virus endemic to Africa, India and Southeast Asia and transmitted by *Aedes* mosquitoes, mainly members of the subgenera *Aedimorphus*, *Diceromyia* and *Stegomyia* [6]. In Europe, it was first recorded in Albania in 1974 [7], and the most recent outbreak occurred in north-eastern Italy in the province of Ravenna during the summer of 2007. About 200 human cases with high fever, arthralgia, myalgia, severe headache and rash were recorded [8]. *Aedes albopictus* was considered to be the most likely vector for this outbreak. An additional potential problem is the capacity of this mosquito to transmit other arboviruses, e.g. dengue virus. For this reason, arboviruses previously believed to cause only tropical diseases must now be considered as potential agents of disease in Mediterranean countries [9].

The main sources of intercontinental movements are migration, political refugees, cooperating persons, and international adoptions. Throughout history, there are many examples of the role of missionaries, explorers, wanderers or other types of travellers who introduced a human pathogen into a susceptible population. Population movements associated with wars or violent conflicts, as well as environmental disasters, can lead to epidemiological outbreaks (e.g. cholera and typhoid fever), which are the consequence of population overcrowding, malnutrition, unhygienic conditions, and basic medical services. One of the infectious diseases most frequently associated with these situations is cholera.

Migrations are constituted by persons who go to a country or region and stay for a long time. The 20th and 21st centuries have witnessed the migration of multiple populations due to war, civil unrest, ethnic cleansing, genocide, economic migration, and geographical catastrophes [3]. At the end of the 20th century, approximately 150 million people lived elsewhere than their native country (2.5% of the world's population); of these, about 15 million were refugees.

Migration of large numbers of people creates opportunities for the spread and establishment of common or novel infectious diseases. Diseases that are imported can be classified as: (i) cosmopolitan imported diseases, when they are distributed worldwide; and (ii) tropical imported diseases, when they exist in tropical and subtropical areas but are nearly non-existent in developed countries because of the lack of an adequate vector or eradication. Roca *et al.* [10] analysed 1321 African immigrants in Spain, and the cosmopolitan imported diseases most frequently found were ancylostomiasis, HIV disease, chancre, tuberculosis, gonorrhoea, and diarrhoea caused by *Giardia lamblia*. Among the tropical imported diseases that they found were schistosomiasis,

intestinal amoebiasis, and malaria. Cosmopolitan diseases have a greater likelihood of increasing their incidence than tropical diseases, because cosmopolitan diseases may have vectors previously adapted to the environment.

International travel, tourism and commerce are increasing, and they constitute an efficient transport system for pathogens and vectors [4]. The introduction of budget and low-cost airlines has revolutionized the travel industry, making travel significantly cheaper [5]. The World Traveller's Organization reported that the destination of about 40% of intercontinental journeys is an underdeveloped country. In 2000, 79 million people chose an exotic country as their destination for holidays. The SARS outbreak is an example of how travel aided the rapid spread of an emerging pathogen from China to susceptible populations worldwide [3]. Tourists risk acquiring infections when they are in new environments, because they are exposed to illnesses for which they have no resistance. Traveller's diarrhoea caused by different diarrhoeagenic *Escherichia coli* strains is one of the most common infectious diseases imported from exotic countries.

Antimicrobial resistance has been considered to be an EID. The easy acquisition of antibiotics and their incorrect and abusive use play an important role in the increase of bacterial resistance to these antimicrobial agents. In addition, resistance can be spread to bacteria belonging to the same or different species by mobile genetic elements such as plasmids, transposons, or integrons.

Finally, the role of environmental change, a global problem, is important in dissemination of infectious diseases. There are multiple ways by which environmental changes could contribute to the emergence and spread of infectious diseases: natural disasters (e.g. flood, drought, earthquake, and famine), deforestation, global warming, and climate change [1]. Deforestation, road construction, irrigation, intensive agriculture and animal production systems, uncontrolled spread of human habitat, lack of public health and contamination are examples of environmental changes caused by human activity that facilitate the emergence of infectious diseases. Zoonotic transmission of infectious diseases is facilitated by encroachment of human habitation and the loss of niche environments [3]. However, the pathogen must first establish itself in the new environment, and pathogens that need a vector or an intermediate host will have a restricted distribution, depending on vector or host adaptation to, or availability in, the new environment. Non-zoonotic means of transmission, such as person-person or faecal-oral transmission of infectious diseases, can be affected by living conditions, size or density of population, sexual habits, and population levels of immunization. In addition, human behaviour has contributed to a number of EIDs within different

populations. The most recognized is the role of sexual behaviour in the transmission of sexually transmitted diseases, such as HIV disease. Alcoholism, body piercing, tattooing, intravenous drug abuse and changes in eating habits have also been responsible for EIDs.

For a country to defend itself against the emergence and spread of infectious diseases, it must have a surveillance system to detect emergent and re-emergent infections, responsive healthcare systems and laboratories, vector control, and education programmes to inform the population of how to avoid infections.

### Transparency Declaration

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# The impact of climate on the disease dynamics of cholera

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## Abstract

The size of infectious disease outbreaks frequently depends on climate influences as well as on the level of immunity in the host population. This is particularly the case with vectorborne and waterborne diseases, for which pathogen transmissibility critically depends on ecological conditions. Here, a mathematical model that was applied to the bacterium *Vibrio cholerae* to understand its disease dynamics in Bangladesh is reviewed. When interfaced with empirical case data on cholera, the model shows that climate plays a pivotal role in modulating the size of outbreaks, with local, regional, and global indices of climate variability showing a link with pathogen transmissibility. Furthermore, the incidence of cholera may occasionally be surprisingly low at times when climate seems to favour cholera transmission.

**Keywords:** Bangladesh, cholera, climate, immunity, infectious disease

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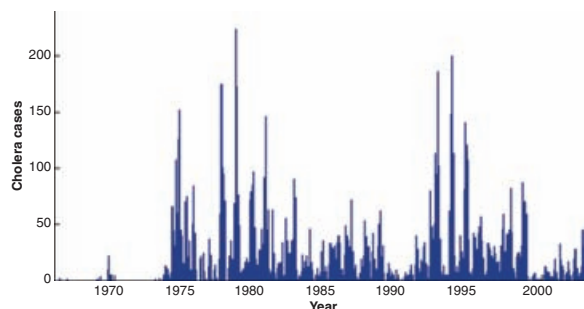
Many infectious diseases are characterized by epidemic outbreaks that vary in size, both seasonally and interannually. For example, malaria incidence in the highlands of East Africa is known to peak every summer, with larger outbreaks occurring every 3 years [1]. Similarly, dengue outbreaks in Thailand occur annually between June and October [2], with larger outbreaks occurring every 2–4 years [1,3]. As with malaria and dengue, cholera dynamics in Bangladesh also show evidence of seasonal and interannual variability: more individuals become infected with the bacterium during the spring and during the autumn than during the summer monsoon season or the dry winter [4], and outbreaks are much larger in some years than in others [5](Fig. 1).

To ultimately predict when and where outbreaks of these infectious diseases will occur, it is necessary to understand the factors contributing to these seasonal and interannual patterns in disease incidence. In the case of vector-borne and waterborne diseases, one critical factor affecting the patterns in disease incidence is climate variability. For vector-borne diseases, temperature and rainfall levels are known to affect mosquito development times and viral amplification rates [6]. These rates in turn affect the transmission rate of vector-borne pathogens, such as those causing malaria and dengue. In the case of waterborne diseases, climate can affect standing water levels, as well as standing water proper-

ties, including salinity, temperature, ionic content, and resident biota. These factors can affect pathogen survival rates in their aquatic reservoir [7], and thereby also affect the pathogen's transmission rate.

Although it is clear that climate variability can affect disease dynamics, which climate variables are most relevant to the dynamics, and at which scales these climate variables act, are difficult to ascertain. This is primarily because we do not have information on how pathogen transmission rates vary in time. Instead, in the best case scenarios, we know only the magnitude of case outbreaks over time. These temporal fluctuations depend only partially on the transmission rate of the pathogen; they also depend on the non-linear interaction between infected individuals and the susceptible host population. For example, a period of low disease incidence may be a result of low transmission rates, or it may be a result of a low number of susceptible individuals in the population (because of previously occurring disease outbreaks). Owing to this confounding factor of immunity in the population, the role of climate cannot be easily isolated by correlations with disease incidence [8].

Instead of attempting to identify the role of climate through simple correlation with disease incidence, we must adopt more sophisticated approaches to identifying climatic drivers. These approaches include the development of mathematical models and statistical non-linear time-series analyses. Specifically, the mathematical models must explicitly allow for temporal fluctuations in herd immunity levels and provide a mechanism for including the effects of climate variability. In this article, I discuss the formulation of a previously published compartmental mathematical model [9] and its sta-

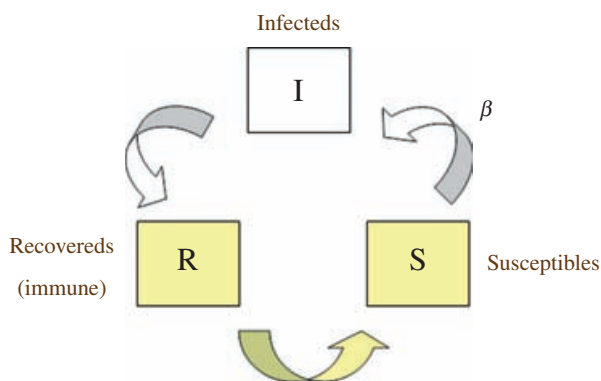


**Fig. 1.** Interannual variability in the size of cholera outbreaks in Matlab, Bangladesh. Only cholera cases of the El Tor strain are shown. Reproduced from [5].

tistical fit to cholera incidence data [5] (Fig. 1). The disease dynamics of cholera are modeled with a difference equation of the form:

$$I_{t+1} = \beta_t I_t \frac{S_t}{N_t},$$

where  $I_t$  is the number of infected individuals,  $S_t$  is the number of susceptible individuals,  $N_t$  is the population size, and  $\beta_t$  is the transmission rate of the pathogen, all at time  $t$ . The schematic of this mathematical model is shown in Fig. 2. According to this model, infected individuals ( $I$ ) transmit the bacterium to susceptible individuals ( $S$ ), at a rate  $\beta$ . (Division by the population size,  $N$ , simply rescales the transmission rate,  $\beta$ , and makes the model statistically more tractable.) Fluctuations in herd immunity are captured by variation in  $\frac{S_t}{N_t}$  over time. The effect of climate variability on cholera dynamics is captured in fluctuations in the transmission rate,  $\beta$ , over time. This model is sufficiently general in form to be applied to other infectious diseases. In addition to this equation, which determines the number of infected individuals over time, the model requires another equation that tracks



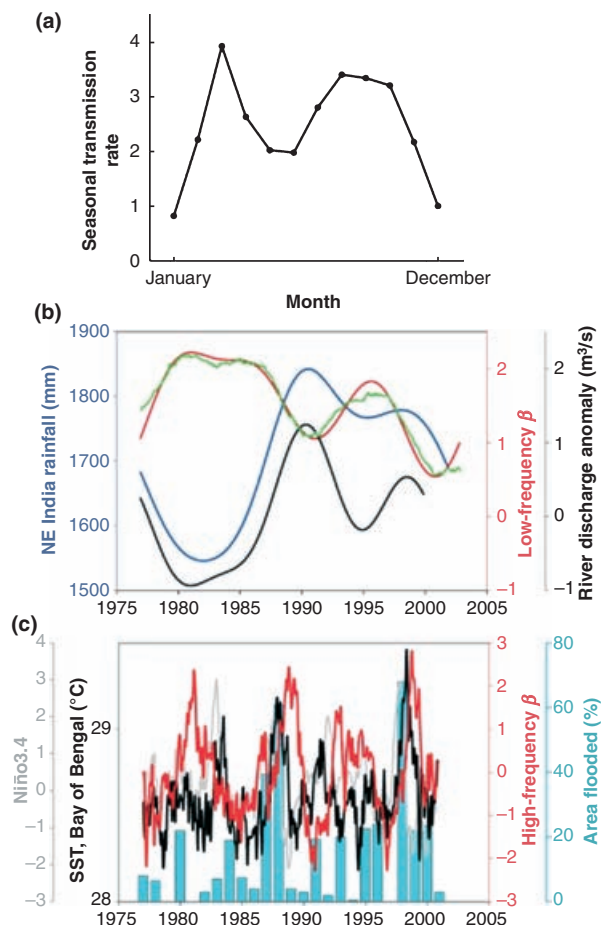
**Fig. 2.** A schematic of the mathematical model applied to cholera dynamics, described in detail in [9].

how the number of susceptible individuals changes over time. More specific details on the model formulation can be found in [9] and [5].

The results of fitting this model to the time series shown in Fig. 1 clearly indicate that, once fluctuations in herd immunity are taken into account, a strong signal of climate forcing is seen in the dynamics of cholera outbreaks. Our results, described in [5], show that cholera dynamics are affected by climate variability at multiple different scales (local, regional, and global). First, we found that the seasonal variation in transmission levels was clearly associated with the timing of the monsoon that hits Bangladesh locally: transmission rates of the bacterium were low during the summer rains and the dry winter, and peaked in the spring and in the autumn (Fig. 3a). Second, we found that long-term variation in transmission rates had an inverse correlation with Brahmaputra river discharge anomalies and northeast India rainfall levels (Fig. 3b). These regional climate influences therefore play a modulating role at interannual time-scales. Third, we found that the short-term unexplained variation in cholera transmission rates (assumed to be statistical noise in the model) showed a significant 8–10-month lagged correlation with the global climate index ENSO, the El Niño Southern Oscillation (Fig. 3c). ENSO has previously been shown to affect cholera dynamics through the use of statistical methods, such as scale-dependent correlation [10], which identify strong local correlations between two time series (here, climate variability and disease incidence).

In addition to finding evidence for climatic effects on cholera dynamics in Bangladesh, this research identified an important interaction between herd immunity fluctuations ( $\frac{S}{N}$ ) and climate forcing (affecting transmission,  $\beta$ ). During certain times of moderate or even low cholera incidence, climate indices and reconstructed transmission rates indicated strongly favourable conditions for the spread of cholera. To understand the reasons for the occurrence of this phenomenon, we looked more closely at herd immunity levels during these periods. We found that cholera outbreaks could not occur, even in the presence of favourable climate conditions, if a larger outbreak had occurred within the previous several years. This is because, following a large outbreak, levels of herd immunity remain high, such that the system would be in a 'refractory period', unable to respond to any external climate forcing, owing to the lack of susceptible hosts in the population. A clear example of a refractory period occurred in 1986–1987, following the prolonged cholera outbreaks occurring between 1977 and 1982.

In sum, to understand the factors contributing to the seasonal and interannual variation in the size of infectious disease outbreaks, it is necessary to disentangle the role of



**Fig. 3.** The effect of climate on the transmission of cholera. (a) Statistical fit of cholera's seasonal transmission rates in Bangladesh. Peaks of transmission occur in the spring and in the autumn. Transmission troughs occur during the summer monsoon and during the dry winter. (b) Regional climate variables (northeast India rainfall (blue) and Brahmaputra river discharge levels (black)), and their inverse correlation with long-term cholera transmission rates (green and red). (c) Niño3.4 (grey), a global climate index for ENSO, and its positive lagged correlation with cholera transmission of high frequency (red). Cholera transmission lags Niño3.4 by 8–10 months. Also shown are the lagged correlation between Niño3.4 and sea surface temperature (SST) in the Bay of Bengal (black), and the percentage of Bangladesh flooded (turquoise bars). The effect of Niño3.4 on cholera dynamics may act through more local variables such as SST and flooding. Subplots a–c reproduced from [5].

climate from the role of herd immunity fluctuations. Understanding their separate effects, as well as their interaction, is critical for the development of effective early-warning systems for infectious disease outbreaks.

## Transparency Declaration

The author declares no conflicts of interest.

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# Adaptive drug resistance mediated by root-nodulation-cell division efflux pumps

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## Abstract

Bacterial resistance to antibiotics is a major therapeutic problem. Bacteria use the same mechanisms for developing resistance to antibiotics as they do for developing resistance to biocide compounds present in some cleaning and personal care products. Root-nodulation-cell division (RND) family efflux pumps are a common means of multidrug resistance, and induction of their expression can explain the observed cross-resistance found between antibiotics and biocides in laboratory strains. Hence, there is a relationship between the active chemicals used in household products, organic solvents and antibiotics. The widespread use of biocide-containing modern-day household products may promote the development of microbial resistance and, in particular, cross-resistance to antibiotics.

**Keywords:** Antibiotic resistance, biocides, efflux pumps, *Pseudomonas*, RND

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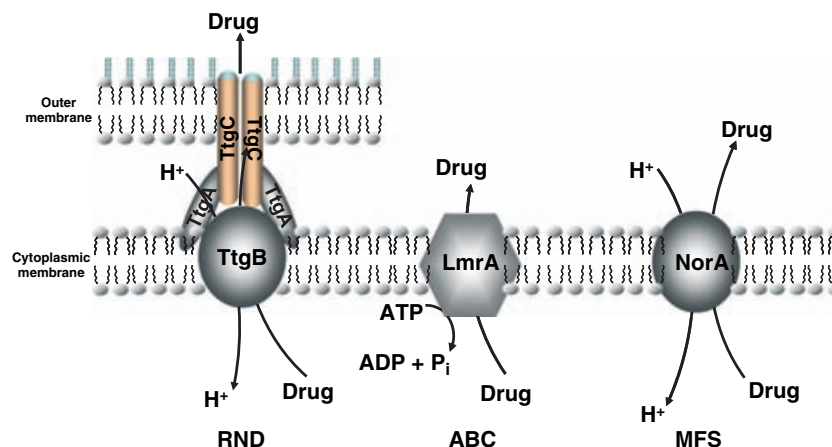
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Since the first high-yield production of penicillin, antibiotics have had a profound impact on human health and society. The recent development of personal care and household cleaning products that contain antibacterial compounds has influenced personal hygiene. The former may have contributed to preventing microbial infection on the skin of individuals, and the latter have helped to combat microbes in the environment. The widespread use of antibiotics, and personal care/cleaning products that contain antibacterial agents, has created an optimistic view that many potential bacterial infections can be defeated. The indiscriminate use of these products, however, has resulted in selective pressure enhancing the development of antibiotic resistance in microbes. As a consequence, drug-resistant pathogens are becoming increasingly pervasive [1]. The resurgence of tuberculosis and other infectious diseases exemplify the risks associated with evolving drug resistance.

Four strategies that lead to antimicrobial drug resistance are commonly used by bacteria and other cells: (i) the target enzyme of an antibiotic may mutate and acquire resistance; (ii) new enzymes may evolve that degrade the antibiotic; (iii) entry of antibiotics might be excluded or limited because of cell membrane modifications; and (iv) the antibiotic may be exported from the cell [2].

Multidrug resistance (MDR) is a phenomenon whereby a host displays intrinsic resistance to a broad range of chemicals against which it has never previously been exposed. Specifically, MDR efflux transporters actively extrude chemicals out of the cell with a remarkably broad range of substrate specificity, and are typically most effective when combined with other resistance mechanisms [3]. Thus, chemical agents are effectively prevented from reaching concentrations lethal to the host.

MDR efflux transporters have been identified in eukaryotes, bacteria and archaea, and are classified into five major groups [4] (Fig. 1): (i) the ATP-binding cassette transporters, which couple the hydrolysis of ATP to the export of substrate; (ii) the major facilitator superfamily transporters; (iii) the small multidrug resistance family transporters; (iv) the root-nodulation-division (RND) family transporters, all of which couple the energy stored in a transmembrane proton gradient to export substrate; and (v) the multi-antimicrobial extrusion family, which couples either a proton or sodium gradient to export substrate. In Gram-negative bacteria, which contain both inner (cytoplasmic) and outer membranes separated by a periplasmic space, the outer membrane does not support a protonmotive force. Thus, bacterial RND transporters utilize the protonmotive force across the cytoplasmic membrane to extrude substrate. The complex is typically composed of an energy-coupled integral membrane protein pump associated with an outer membrane protein that enters into the periplasm and produces an exit duct, and a third element that acts as an adaptor and guarantees its structural integrity [5]. Therefore, bacterial RND efflux systems span the periplasm and both inner and outer



**Fig. 1.** Illustration showing the main types of bacterial efflux systems. Indicated are TtgABC from *Pseudomonas putida* (a member of the root-nodulation–cell division (RND) family), LmrA from *Lactococcus lactis* (a member of the ATP-binding cassette (ABC) family) and NorA from *Staphylococcus aureus* (a member of the major facilitator superfamily (MFS) family). As mentioned in the text, there are two other main types of efflux systems found in bacteria, small multidrug resistance (SMR) and multi-antimicrobial extrusion (MATE), and both systems appear to be structurally similar to the MFS family of pumps.

membranes, thus clearing toxic chemicals from intracellular compartments.

Numerous recent studies have indicated that the use of antimicrobial household cleaning agents can select for organisms with adapted efflux pump resistance mechanisms [6–9]. *Pseudomonas aeruginosa* is normally resistant to the bisphenol compounds that are often added to home cleaning and hygiene products; the resistance is brought about by the active efflux of the compounds by the MexAB–OprM RND family efflux pump. Chuanchuen *et al.* have shown that *P. aeruginosa* strains lacking the MexAB–OprM pump can be selectively pressured by bisphenol compound exposure to hyperexpress another RND efflux pump family member (MexCD–OprJ) and subsequently become multidrug-resistant [7,8]. Further studies using the human enteric pathogen *E. coli* O157 have shown that sublethal exposure to quaternary ammonium compounds and bisphenols can result in adaptive resistance to various antimicrobial agents, including tetracycline, chloramphenicol and biocides [6]. In *E. coli*, the proposed mechanism of bisphenol resistance and cross-resistance is the overexpression of the active efflux pump AcrAB [9]. In a more recent study, using the reverse hypothesis that antibiotic-resistant organisms become tolerant to other antibacterial agents owing to the action of efflux pumps, Thorrold *et al.* implicated efflux pump activity in *Salmonella* and *E. coli* in causing reduced susceptibility to household antimicrobial cleaning agents [10]. A promising strategy for addressing MDR in Gram-negative bacteria, therefore, aims to counteract RND efflux pumps, potentially halting the clearance of drugs from the cellular milieu.

Many pathogenic and non-pathogenic microorganisms, such as *P. aeruginosa*, *E. coli*, *Salmonella* spp. and *Pseudomonas putida*, can grow in the presence of selected solvents and antibacterials; in some extreme cases, as with *P. putida* DOT-T1E, growth can reach high cell densities in even 1% (v/v) of a number of linear, cyclic and aromatic hydrocarbons and other compounds (e.g. organic alcohols and phthalates), many of which are present in available household cleaning products [11].

*P. putida* DOT-T1E is therefore an interesting model system, and several laboratories have established that three general mechanisms are involved in the tolerance of this strain to toxic chemicals [12,13]. One of these mechanisms is a set of physical barriers intended to increase the rigidity of the cell membrane. In particular, the bacterium responds by altering the *cis* to *trans* ratio and by modifying the phospholipid headgroups [14–17]. Second, the bacterium has an exquisite set of tools ready to respond to unexpected situations, and uses a kind of general stress response involving a series of chaperones and DNA repair systems to overcome toxic effects [18–20]. The third mechanism, probably the most important, involves the series of efflux pumps that remove toxic chemicals from the cell. An important point is that tolerance to toxic chemicals and antimicrobials can be greatly influenced by the conditions under which bacteria are grown. For instance, when *P. putida* strains are grown in the absence of the solvent toluene and are then suddenly exposed to 0.3% (v/v) toluene, only a small fraction of the cells, about one in 10 000, survive the shock [21]. However, if the strains are precultured in the presence of low concen-



trations of toluene, 50–100% of the cells survive a subsequent solvent shock. These results suggest that some aspects of solvent tolerance involve the induction of multiple solvent tolerance determinants [22].

To investigate the role of different elements in solvent tolerance and antibiotic resistance, our research group looked for solvent-sensitive mutants via generalized mini-Tn5 mutagenesis. One such mutant is *P. putida* DOT-TIE-18, which, in contrast to the wild-type strain, was not able to tolerate a sudden toluene shock and also showed significantly diminished induced tolerance [21]. We also tested this strain for antibiotic tolerance, and found a concomitant increase in antibiotic sensitivity [23]. Sequencing of the DNA adjacent to the mini-Tn5 insertion revealed that DOT-TIE-18 had a knockout in an operon that encoded a solvent exclusion RND efflux pump, which we called TtgABC. This pump is expressed at a basal level from a single promoter when the bacteria are grown under laboratory culture conditions [24]. The proteins TtgABC consist of the pump itself (TtgB), located in the inner membrane, and a porin (TtgC) that crosses the outer membrane and penetrates through the periplasm to reach the pump [5]. This outer membrane protein generates a channel through which toxic chemicals are extruded. The interactions between TtgB and TtgC are maintained by a lipoprotein (TtgA) that is fixed to the inner membrane [25].

Expression of the *ttgABC* operon is controlled by TtgR (Fig. 2); *ttgA* and *ttgR* are transcribed divergently and, based on the determination of their corresponding transcription start points, their promoters overlap [24]. Recently, we purified TtgR to homogeneity in our laboratory and found that TtgR is a dimer in solution and is able to bind to the intergenic region between *ttgR* and *ttgA*, as shown by electro-

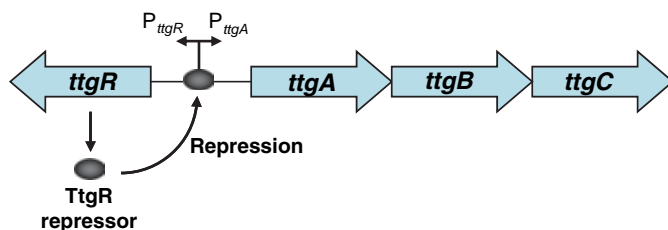
phoretic mobility shift assays (EMSAs). DNaseI footprints revealed that TtgR protected four helical turns in the promoter region; more specifically, it bound a 30-bp operator. The stoichiometry of TtgR binding to target DNA was determined by carrying out a series of equilibrium centrifugation analyses, which revealed that one dimer was bound per operator [26].

Using isothermal titration calorimetry, Krell *et al.* [26] showed that TtgR binds with relatively high affinity (100 nM) to its target operator. The sequence of the operator to which TtgR binds revealed that the target could be an overlapping inverted repeat.

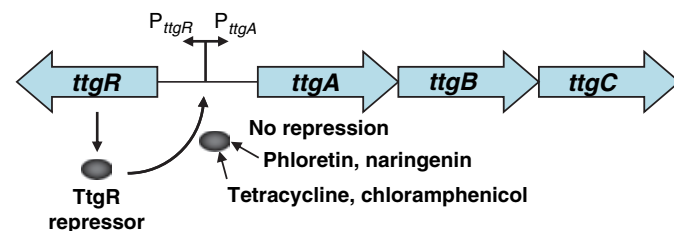
A series of early competition assays revealed that the TtgABC efflux pump removes toluene, xylene, styrene, naphthalene and other aromatic compounds [11]. Terán *et al.* [23,27,28] have shown that the efflux pump also removes antibiotics and flavonoids. Subsequently, using EMSA, we tested whether the induction of the efflux pump occurred in response to the compounds extruded by the pump. Our rationale was that in the absence of effectors, TtgR binds and retards DNA. If TtgR interacts with a drug, the protein should be released and DNA should not be retarded. Among the TtgR effectors there are antibiotics (chloramphenicol, tetracycline and ampicillin) and flavonoids (naringenin and phloretin), but not hydrocarbons [27].

The affinity of TtgR in solution and bound to DNA for its effectors was determined by microcalorimetric titration with free and DNA-bound protein. An extensive series of kinetic analyses allowed Krell *et al.* [26] to determine that the affinity was in the low micromolar range. Alguel *et al.* [29] also established, through equilibrium analysis, that each TtgR dimer binds one effector molecule both in solution and when bound to DNA.

**(a) The *ttgABC* operon is not transcribed when TtgR is bound to the promoter**



**(b) The *ttgABC* operon is transcribed in the presence of antibiotics and flavonoids**



**Fig. 2.** Regulation of *ttgABC*, a root-nodulation–cell division (RND) family efflux pump from *Pseudomonas putida* DOT-TIE. (a) When a wild-type strain is grown in the absence of effector molecules such as antibiotics, the TtgR protein is produced and binds to the promoter/operator site, thus preventing further transcription and expression of the *ttgABC* operon. (b) In the presence of flavonoids and antibiotics, the repression is removed because effector molecules are able to bind to the TtgR repressor and prevent binding of the repressor to the promoter/operator region.

The TtgR protein, which belongs to the TetR family of regulators, has recently been crystallized alone and with several different effectors [29]. It has a highly conserved structure and, like TetR [30] and QacR [31], is composed of nine  $\alpha$ -helices, with  $\alpha_2$  and  $\alpha_3$  constituting the HTH DNA-binding domain. Co-crystallization of TtgR with effectors revealed that the binding pocket of this regulator has a wide cavity that allows it to interact with a multitude of effector molecules by establishing different van der Waals and ionic bridges with numerous amino acids in the effector binding pocket [29]. It was shown that phloretin binds with high affinity to residues His114, Arg130 and Arg176. Tetracycline and other flavonoids, however, bind with low affinity in a different binding pocket. We have recently generated eight independent mutations in residues involved in the binding pocket. We have purified each of the mutant proteins and conducted EMSAs. We found that some of the amino acid changes in this region of TtgR result in a marked increase in affinity for the operator DNA and a subsequent decrease in affinity for certain effector molecules (C. Daniels, unpublished results), suggesting that we have identified the effector-binding pocket.

In summary, multidrug efflux pumps are able to extrude a wide range of chemicals and can be specifically induced in response to certain compounds. These traits can lead to an increase in the level of resistance to all drugs extruded by the pump. Therefore, the use of non-antibiotic chemicals in household cleaning products may influence the efficiency of hospital drugs, both biocides and antimicrobials, because of acquired and induced resistance.

## Transparency Declaration

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# Tracking human migration patterns through the oral bacterial flora

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## Abstract

As modern humans (*Homo sapiens*) migrated out of Africa to different parts of the world, their obligate indigenous bacterial biota accompanied them. As both evolved, the accumulations of mutations in their DNA can reveal their phylogenies. Here, we describe the evolutionary history of an indigenous bacteria, *Streptococcus mutans*, from the oral cavity. Using several genetic markers, four distinct clusters of *S. mutans* genetic traits coincide with individuals of distinct geographic or racial groups comprised of two African clades and an Asian and a Caucasian clade. The evolutionary lineage of *S. mutans* is in agreement with anthropological artifacts marking the trail of human migrations.

**Keywords:** Co-evolution, homo sapiens, intergenetic spacer region, phylogeny, plasmid, *Streptococcus mutans*

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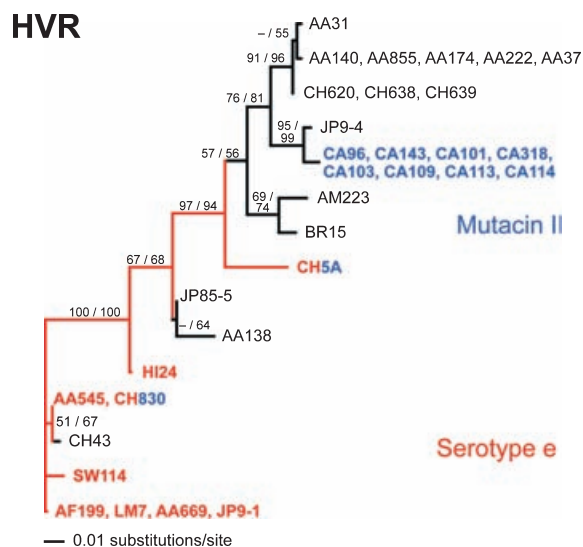
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The oral flora is composed of hundreds of different genotypes of bacteria. Some have been cultivated, and about 40% remain uncultivated [1]. The most common form of childhood infection worldwide is tooth decay (i.e. dental caries). Mutans streptococci (MS) have been implicated as major contributing factors in the initiation of dental caries in childhood. However, the MS are members of the normal indigenous biota of humans, being present in both caries-active and caries-free individuals, and thus are essentially ubiquitous in dentate humans. The MS are acquired in infancy, mostly from the mother during a period known as the window of infectivity [2]. Colonization is stable and probably lifelong, with the average adult human harbouring two genotypes.

The indigenous biota of humans, or the 'second genome', including the MS, are thought to have co-evolved with their human hosts. Support for this notion of co-evolution was recently obtained through examination of the phylogeny of plasmid-containing strains of *Streptococcus mutans* and their discrete clustering within racial and geographically defined populations [3]. Supporting a concept of host–parasite co-evolution is the small genome size of *S. mutans* as it undergoes parasitic degeneration, which is common among obligate parasites. The ubiquitous distribution of MS in humans further suggests that MS are indigenous to humans,

as does the observation that *S. mutans* is mainly transmitted vertically, and only from mothers to offspring, not from fathers, suggesting that this mode of transmission might be reflective of an overall theme, i.e. conservation of genotypes or strains along maternal lines. In fact, the mitochondrial DNA from only maternal sources was among the first genetic evidence used to document human migrations out of Africa [4]. Could mother–child transmission be expanded on a larger scale to reflect evolutionary histories of the human host and its indigenous biota?

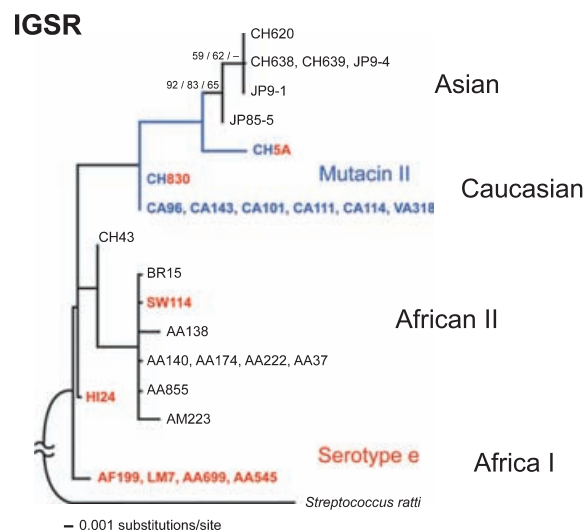
From the evidence for co-evolution between *S. mutans* and human hosts, we wanted to reconstruct the evolutionary history of *S. mutans* isolated from different racial and ethnic populations, to determine whether strains cluster or show clonality within human populations. To do this, we used a polyphasic approach to phylogeny, using a combination of genetic traits and DNA sequence to reconstruct the most parsimonious history of *S. mutans*, and then determining whether strains cluster within racial/ethnic groups from different geographical locations. We chose strains of *S. mutans* that contained plasmid DNA, which limited the clonal size of the population and, as it later turned out, indicated that these strains were genetically more diverse than their plasmid-free counterparts. Strains of *S. mutans* were obtained from five continents, including sampling from Brazil, Guyana, the USA, Africa, China, New Zealand, Australia, and Japan. In total, 36 plasmid-containing strains comprised the sample. Genetic loci indicating mutacin type and serotype were identified for each strain. Strains were also subjected to chromosomal DNA fingerprinting to ascertain genotypes.



**Fig. 1.** Phylogeny of the cryptic 5.6-kb plasmid from *Streptococcus mutans*. Strains in red are serotype e and are shown to exhibit a common evolutionary derivation, whereas racial/geographical host and mutacin gene distribution are not correlated with the plasmid's history.

The first comparison that we performed was with DNA sequences from the hypervariable region of the plasmid, to determine whether plasmid phylogeny paralleled that of human populations. A 600-bp hypervariable region of the plasmid was chosen because, like the hypervariable region of the mitochondrial genome, it had the greatest level of polymorphic and informative sites (approximately 10% of the sites were variable). After alignment, a phylogenetic tree (Fig. 1) was constructed, using a maximum-likelihood algorithm followed by bootstrap analysis for robustness of the final tree. As seen in Fig. 1, a well-supported tree that recapitulated the evolutionary history of the plasmid resulted. However, the derived tree did not reflect the evolutionary history of its human host; rather, the history of the plasmid was strongly suggestive of horizontal gene transfer, as would be expected for other mobile genetic elements.

A second phylogenetic tree was constructed from the intergenetic spacer region between the 16S and 23S rRNA genes. The fragment was first amplified by PCR, and then sequenced. Only a few sites were phylogenetically informative, and they were insufficient to construct a well-supported tree using maximum likelihood. Using a polyphasic approach, other genetic loci such as serotype and mutacin type were added to the analysis, each site being weighted as either present or absent, and various weighting procedures evaluated using the Model Test algorithm. The intergenetic spacer region of *Streptococcus ratti* served as the outgroup. A tree results, in which racial/geographical populations appear to



**Fig. 2.** Phylogenetic tree depicting co-evolutionary histories of *Streptococcus mutans* and its human host. Clustering and similarity of genotypes of Caucasian strains suggest an Asian ancestor and small founder population.

cluster, particularly the Asian cluster, which is well supported by bootstrap analysis (Fig. 2). In addition, the African I cluster, which contains strain AF199, is positioned proximal to a theoretical common ancestor, supportive of the 'out of Africa' hypothesis [4]. Our tree resembles that derived from multiple genetic loci from *Helicobacter pylori*, another putative member of the human indigenous biota [5].

In summary, plasmid and chromosomal evolutionary histories are different, suggesting two levels of host-parasite relationship, one between plasmid and *S. mutans*, and one between *S. mutans* and its human host. That the cryptic plasmid's phylogeny did not parallel the evolutionary history of *S. mutans* or that of human populations was not surprising, given the known propensity for horizontal gene movement of mobile, self-replicating units of DNA. The clustering of serotypes with plasmid clusters suggests that the serotype locus may have been acquired with the plasmid. Mutacin loci did not appear to be correlated with plasmid clusters, but more evidence is needed before drawing conclusions, especially given that all plasmid strains elaborate at least one mutacin type. The bacteriocins of the sister genera *Lactobacillus* and *Lactococcus* are often plasmid-encoded. Both trees support an African root. Additional polymorphic loci are needed to more definitively resolve the evolutionary history of *S. mutans* and to further delineate population differences in its human hosts. Overall, the evolutionary history of *S. mutans* suggests that as early humans migrated out of Africa, they were accompanied by their obligate indigenous co-evolved companions.

## Transparency Declaration

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# Biodiversity loss and the rise of zoonotic pathogens

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## Abstract

The unprecedented loss of biological diversity from anthropogenic causes has profound impacts on human health. One way that biodiversity loss threatens human health is by exacerbating risk and incidence of infectious diseases. This paper briefly reviews two zoonotic diseases – West Nile virus (WNV) illness and Lyme disease (LD) – in which high diversity in the community of vertebrate hosts for arthropod vectors strongly reduces human risk. In both cases, the primary reservoirs for the pathogen are species that dominate in human-impacted, low-diversity communities. As a result, the generalist vectors responsible for transmitting the pathogens to humans have relatively high feeding rates on these reservoirs, leading to high infection prevalence in mosquito (for WNV) and tick (for LD) vectors. In contrast, where native vertebrate diversity is high, mosquito and tick vectors evidently feed from a wider variety of hosts, most of which are poor reservoirs for the pathogens, resulting in lower infection prevalence. Protection of humans against exposure to zoonotic pathogens should be added to the list of utilitarian functions provided by high biodiversity.

**Keywords:** Dilution effect, ecosystem services, emerging infectious diseases, Lyme disease, West Nile virus

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## Introduction

The earth is currently experiencing an extinction crisis that is unprecedented in both magnitude and pace. Although the ability of scientists to ascertain how many species have or will soon become extinct varies considerably among taxonomic groups and ecosystem types, it is clear that the current extinction crisis is pervasive, even universal. At current rates, global extinctions within some classes of vertebrates are predicted to approach 50% within about 100 years [5]. Even more pervasive, yet more poorly documented, are local extinctions of populations and metapopulations. The consequences of this loss of biodiversity for humanity are certainly going to be profound [4].

Many species produce chemical compounds that are usable or adaptable as pharmaceutical agents to the direct benefit of human health. Others provide model systems for biomedical research. Yet others maintain genetic and genomic information vital to improving agricultural productivity and human nutrition [4]. More recently, it has been discovered that high biodiversity can protect human health by reducing the risk of certain infectious diseases [14,15,18].

High biodiversity can buffer against disease transmission by one or more of several mechanisms, including: (i) reducing the population density of an important natural reservoir for pathogens; (ii) reducing the population density of arthropod vectors for pathogens; and (iii) reducing encounter rates between vectors and reservoirs or among reservoirs [7]. The phenomenon by which high diversity reduces disease risk, termed the dilution effect [13,14,18], has been best studied in zoonotic diseases. This article briefly describes two case studies, West Nile virus (WNV) and Lyme disease.

## WNV

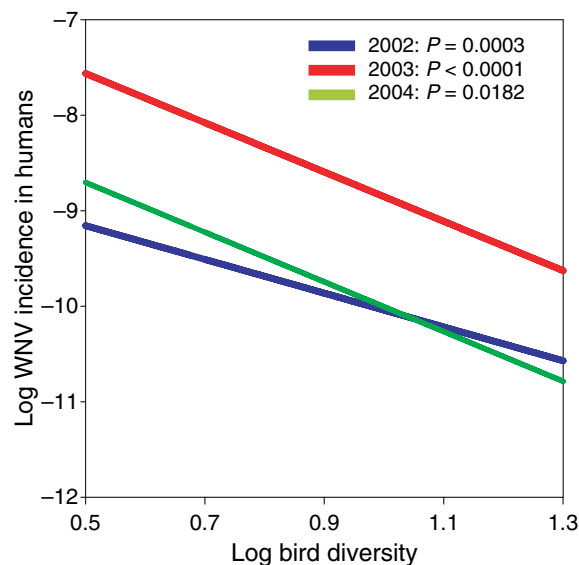
WNV is a mosquito-borne flavivirus that causes substantial morbidity and mortality in several vertebrate groups [9] including humans [10]. The predominant vectors are *Culex* mosquitoes, although other genera, including *Aedes*, can be vector-competent. The predominant reservoirs are passerine birds, although substantial variation exists among species [8,10]. Historically, WNV was restricted to eastern Europe, the Middle East, and eastern Africa, but it was introduced to the New York City area in 1999. Within several years, the disease had spread to the west coast of North America.

The reservoir status of North American bird species exposed to WNV is still being assessed. However, preliminary data indicate that the most competent reservoirs include the blue jay, western scrub jay, common grackle, house finch,

American crow, house sparrow and American robin [8,10]. All of these species are geographically widespread, common, and highly resilient to anthropogenic disturbances, such as urbanization and suburbanization. As a consequence, these species are expected to predominate in habitats in which avian diversity is low. In contrast, in less disturbed habitats these reservoir species tend to be less common or absent, while the diversity and abundance of non-reservoir species is expected to be considerably higher.

The dilution effect hypothesis would predict that mosquitoes occurring in areas of low avian diversity should have a high probability of encountering a competent reservoir for WNV, and therefore a high probability of acquiring infection during blood meals. In contrast, mosquitoes occurring in areas of high avian diversity should have a higher probability of taking a blood meal from one of the many species that are less competent or incompetent as reservoirs for WNV. Consequently, we [2] predicted that counties in the USA with high avian diversity should have a low human incidence of WNV disease, whereas those with low avian diversity should have a high WNV incidence. We were aware that *Culex* mosquitoes show feeding preferences, particularly for American robins, and that strong preferences would reduce the expected importance of total community diversity as a determinant of mosquito or human infection prevalence [8]. We were also aware that the abundance of competent vectors is a potentially strong contributor to host and vector infection prevalence. Strong impacts of feeding preferences and/or vector abundance would undermine the importance of bird diversity in determining WNV prevalence.

For the continental USA, we [2] calculated bird diversity and abundance at the county level from the USGS Breeding Bird Survey raw data (<http://www.mp2-pwrc.usgs.gov/bbs>). We obtained data on all known human cases of WNV disease within each county from the USGS WNV website (<http://westnilemaps.usgs.gov>), and calculated the *per capita* human incidence of WNV for each county (infected persons in county/total persons in county). We determined the year that each state peaked in human incidence for all states with >15 cases from 1999–2004. All states peaked in human incidence in 2002, 2003 or 2004, as the wave of WNV moved westward across the USA, and so we restricted our analyses to these 3 years. Our primary hypothesis was that WNV incidence should be negatively correlated with bird diversity, as measured by the Shannon index, but we also tested the effects of human population density and a community competence index, as well as assessing the importance of spatial autocorrelation (for details, see [2]).



**Fig. 1.** Relationship between log West Nile virus (WNV) disease incidence in humans and log bird diversity for US counties in the year of peak incidence. The figure is based on multiple regressions that hold constant several other potentially important independent variables, such as human population density and an index of avian community competence. Based on data in Allan *et al.* (unpublished).

For all 3 years, the county-level human incidence of WNV disease was strongly, and significantly, negatively correlated with bird diversity within that county (Fig. 1). Spatial autocorrelation had only a modest effect. Neither of the other two independent variables (human population and community competence index) was consistently correlated with WNV incidence. Therefore, despite the potential for mosquito abundance and feeding preferences to destroy any effect of host diversity, our analysis strongly supported the predictions of the dilution effect hypothesis.

## Lyme disease

Lyme disease is a tick-borne bacterial zoonosis caused by the spirochete *Borrelia burgdorferi*. Both the infection prevalence and abundance of the tick vector are critical to determining human exposure rates, and ecological determinants of risk of exposure to Lyme disease show some similarities to those influencing risk of exposure to WNV. In most of North America, the vector is the blacklegged tick, *Ixodes scapularis*, and the primary reservoirs for *B. burgdorferi* are white-footed mice (*Peromyscus leucopus*), eastern chipmunks (*Tamias striatus*), short-tailed shrews (*Blarina brevicauda*), and masked shrews (*Sorex cinereus*) [3,12]. White-footed mice,

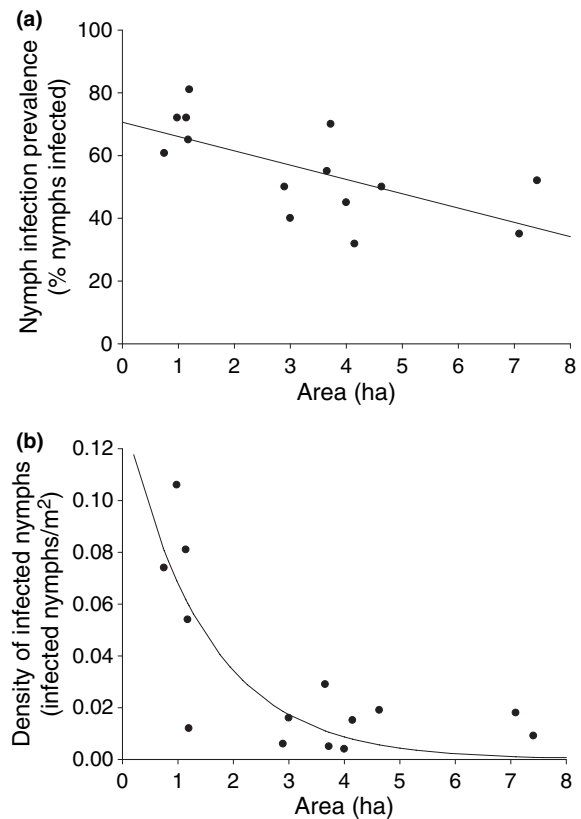
eastern chipmunks and short-tailed shrews are highly resilient, widespread species that are abundant in degraded and fragmented habitats [11,12], and can dominate low-diversity vertebrate communities. Communities with higher mammal and bird diversity contain these species, but also contain many other species that are poor reservoirs for the Lyme disease spirochete. We hypothesized that ticks occurring in forests supporting high vertebrate diversity would have lower infection prevalence than would ticks occurring in low-diversity habitats where mice, chipmunks, and shrews dominate. In addition, because blacklegged ticks appear to feed more successfully from small rodents than from other hosts, we hypothesized that they would be more abundant in low-diversity habitats.

The major factor determining the species richness of terrestrial mammals in some regions is the size of the habitat area [17]. Consequently, we predicted that small forest patches within Lyme disease endemic zones would have higher abundance and infection prevalence of *I. scapularis* ticks. Sampling in 14 forest fragments within a suburban land-use matrix in Dutchess County, New York state, demonstrated a significant negative correlation of both abundance and infection prevalence of nymphal ticks with fragment size [1] (Fig. 2).

Because we have collected extensive information on the reservoir competence of various mammalian and avian hosts, as well as on their average tick burdens and population density, we are able to make specific, quantitative predictions regarding tick infection prevalence given a specific host community [16]. These predictions are possible because, with knowledge of each host species' relative abundance, we can project the number of ticks that will feed on them and the proportion of those ticks that will become infected. We sampled 40 forested sites in the northeastern USA to determine the composition of the host community, and predicted what the tick infection prevalence should be on the basis of our simple models [12,16]. We found a strong, statistically significant correlation between what our models predicted and what we observed in these field sites [11]. We conclude from these studies that high vertebrate diversity is negatively correlated with human risk of exposure to Lyme disease. Furthermore, knowledge of the species composition of these communities, beyond simple measures of species richness or evenness, strongly enhances our ability to predict risk.

## Concluding thoughts

Evidence for a protective dilution effect of high diversity has been obtained for numerous infectious diseases of humans,



**Fig. 2.** Relationship between forest fragment size and (a) prevalence of infection of nymphal ticks with *Borrelia burgdorferi*, and (b) density of infected nymphal *Ixodes scapularis* ticks. Data are from forest fragments in Dutchess County, New York, USA. Reprinted from Allan *et al.* [1].

wildlife, and plants [7]. The weight of evidence suggests that protection against exposure to infectious diseases should be added to the list of utilitarian functions of biodiversity. Biodiversity loss continues to accompany habitat destruction and fragmentation, pollution, invasions by exotic species, and direct human exploitation (e.g. bushmeat hunting and overfishing). However, perhaps the greatest threat to biodiversity in the long term is global climate change. Climate change will therefore probably exacerbate the global burden of disease both directly and indirectly, by reducing biodiversity [6]. For these reasons and more, our health depends on aggressive efforts to reduce the rate of climate change.

## Transparency Declaration

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## Genomics in the detection of damage in microbial systems: cell wall stress in yeast

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### Abstract

*Saccharomyces cerevisiae*, like other microorganisms, has evolved different mechanisms to survive under adverse conditions. The adaptation of yeast to cell wall stress is mainly regulated by mitogen-activated protein kinase (MAPK) pathways. The characterization of genome-wide transcriptional profiles to different cell wall stresses has allowed the identification of those genes important for cell wall remodelling under these circumstances. Moreover, profiling of mutant strains deleted in different elements of these pathways revealed the complexity of the signal transduction machinery responsible for regulating adaptation responses to cell wall stress in yeast. In addition to increase understanding of these adaptive responses, the molecular dissection of these signalling networks could impact on the development of effective new antifungal agents.

**Keywords:** Cell integrity, cell wall, genomics, MAPK, stress, transcriptome

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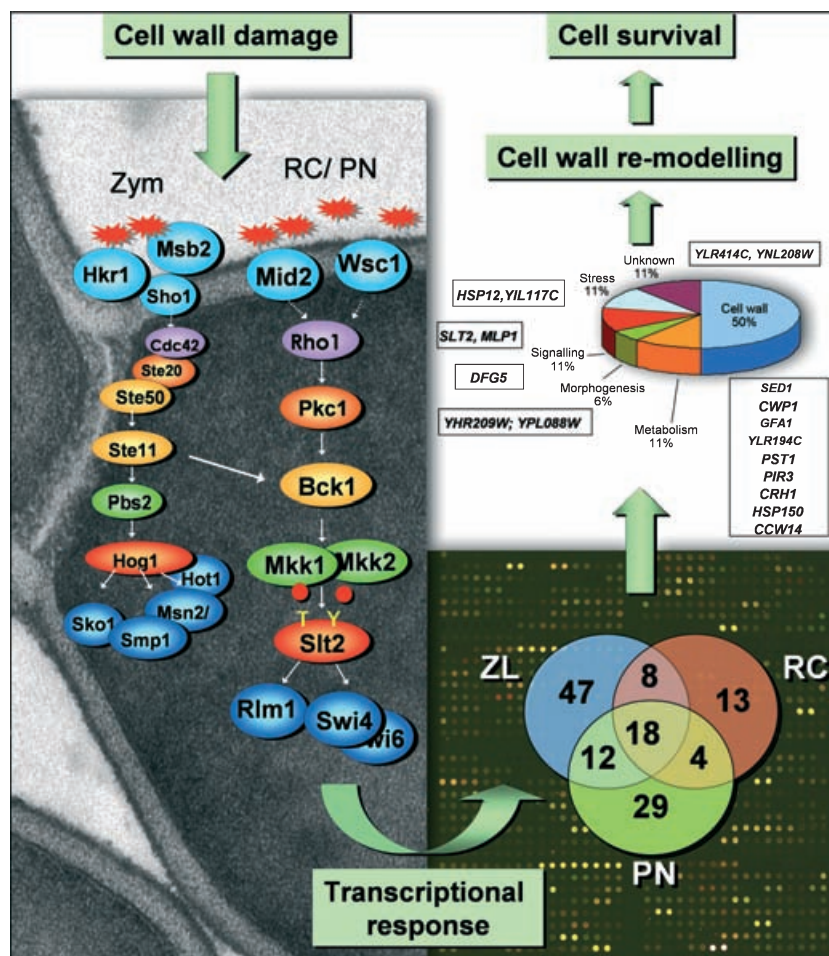
All living organisms, including fungi, use numerous signal transduction pathways to sense the environment, generate adequate cellular responses, and therefore proliferate in a large range of biological niches [1]. There are many stressful conditions in response to which fungal cells have developed sophisticated signalling cascades. In this way, yeast cells can respond to, adapt to and survive many stressful conditions, e.g. osmotic stress, heat, high salt, pH, UV irradiation, oxidative stress, cell wall stress, or stress caused by antifungal compounds. In the yeast *Saccharomyces cerevisiae* (budding yeast), most of these responses are regulated through mitogen-activated protein kinase (MAPK) pathways. This organism has been shown to be an excellent eukaryotic model system with which to extensively study these MAPK signalling pathways. In the budding yeast, four MAPKs—Fus3, Kss1, Hog1, and Slf2/Mpk1—control mating, filamentation/invasion, high-osmolarity, and cell integrity pathways. They are activated in response to mating pheromones, starvation, osmolarity, and cell wall damage, respectively [2].

Yeast cell integrity depends on a particular external envelope, the cell wall, which is a macromolecular complex whose mechanical strength allows cells to support turgor pressure against the plasma membrane [3,4].

Because of the importance of the cell wall for survival, stress conditions that alter this structure lead to the activation of a cellular response named the 'compensatory mechanism' [5] in an attempt by the cell to survive. This response is characterized by: (i) an increase in  $\beta$ -glucan and chitin contents; (ii) changes in the relationship between cell wall polysaccharides; (iii) increases in the amounts of several cell wall proteins; and (iv) the re-localization of important proteins from the cell wall construction machinery to the lateral cell wall.

DNA microarray technology provides a powerful tool for genome-wide transcriptional profile characterization, therefore contributing to our understanding of the molecular basis of stress adaptation responses (Fig. 1). The transcriptional programme of the yeast in response to both constitutive (mutants deleted in genes important for cell wall biogenesis) and transient (presence of cell wall-perturbing agents such as Congo red, zymolyase or pneumocandins) cell wall damage conditions has been extensively studied by means of DNA microarray experiments [6–9]. Analysis of the transcriptional responses to the three different cell wall-interfering compounds named above—Congo red (a compound that binds to chitin, interfering with proper cell wall construction), zymolyase (which affects cell wall integrity as a result of the presence of a main  $\beta$ -1,3-glucanase activity and a residual protease activity), and pneumocandins (inhibitors of  $\beta$ -1,3-glucan synthase)—reveal that the main functional groups of upregulated genes are those involved in cell wall biogenesis, metabolism and generation of energy, signalling, and genes of unknown function. Comparison of the different responses

**Fig. 1.** Yeast cellular responses to cell wall stress. Yeast cells respond to cell wall damage caused by different cell wall stresses (ZL, zymolyase; RC, Congo red; PN, pneumocandin) by activating a transcriptional programme that includes the upregulation of different sets of genes. A common signature of 18 genes, most of them related to cell wall biogenesis, is induced in all these situations. As a consequence of this cellular adaptation response, the cell wall is remodelled for survival. These transcriptional responses are mainly regulated by the CWI pathway, but the HOG pathway is also involved in regulation of responses to stress mediated by zymolyase.



reveals not only the existence of specific transcriptional adaptation profiles for each situation, but also the presence of a common signature of 18 genes that is induced in all these situations (Fig. 1). Interestingly, 50% of this response includes genes directly involved in cell wall remodelling (*SED1*, *CWP1*, *GFA1*, *YLR194C*, *PST1*, *PIR3*, *CRH1*, *HSP150* and *CCW14*). One of the upregulated genes, *CRH1*, codes for a putative transglycosidase activity that, together with the product of the homologous *CRH2*, is involved in the cross-linking between chitin and  $\beta$ -1,6-glucan [10]. Transcriptional activation of *CRH1*, together with the re-localization of the protein encoded by this gene to the lateral cell wall, is part of the mechanism of adaptation to heat stress.

The CWI pathway is the main signalling pathway involved in the regulation of cell wall stress responses. The MAPK of this pathway, Slf2, is encoded by one of the genes transcriptionally induced in the compensatory response. This pathway is activated through several cell membrane proteins (Mid2, Wsc1-4, and Mtl1), with Mid2 and Wsc1 being the main sensors of the pathway. Upon cell wall damage conditions, these sensors interact with the GEF Rom2, activating the small

GTPase Rho1, which then interacts with and activates Pkc1 [4]. The main role of activated Pkc1 is to trigger a MAPK module. Phosphorylation of the MAPK kinase Bck1p by Pkc1 activates a pair of redundant MAPKs (Mkk1 and Mkk2), which finally phosphorylate the MAPK Slf2. The phosphorylated form of this protein acts mainly on two transcription factors: the MADS-box transcription factor Rlm1, and SBF. SBF is a heterodimeric complex of two proteins—Swi4 and Swi6—that is involved in the regulation of gene expression at the G<sub>1</sub>/S transition. As deduced from genome-wide transcriptional studies, activation of the majority of the genes induced in response to Congo red [7], heat shock [11] and zymolyase (R. García and J. Arroyo, unpublished data) is dependent on Rlm1.

Although the cell integrity pathway is the main pathway for cell wall remodelling under cell wall stress, there is also evidence suggesting that different mechanisms are involved in the activation of the MAPK Slf2 under different cell wall stress conditions. Recent data show that the HOG pathway is not only necessary for survival under hyperosmotic conditions but is also involved in adaptation to cell wall stress.

Although cell wall-interfering compounds such as Congo red and zymolyase elicit common genome-wide transcriptional responses related to the set of genes involved in cell remodelling and signalling [7], adaptation responses to these two cell wall stresses are differentially regulated. The response to Congo red depends only on the CWI pathway, whereas the Sho1 branch of the HOG pathway, in addition to the CWI pathway, is also responsible for remodelling the cell wall in response to specific cell wall damage to the  $\beta$ -1,3-glucan network caused by zymolyase. Zymolyase activates both MAPKs, and Slr2 activation depends on the Sho1 branch of the HOG pathway under these conditions. Thus, sequential activation of two MAPKs (HOG and SLT2) pathways is required for cellular adaptation to this specific cell wall stress [12]. Interestingly, zymolyase cell wall stress is not sensed through sensors of the CWI pathway but depends on the mucin-like proteins Hkr1 and Msb2, recently described as putative sensors of the Sho1 branch of the HOG pathway [13]. Furthermore, Congo red and pneumocandins activate the CWI pathway, but the sensors Mid2 and Wsc1 of this pathway seem to play different roles in this activation. Whereas activation by Congo red of Slr2 and the induction of the transcriptional programme by this drug are mainly dependent on Mid2, the adaptation response to pneumocandins is mainly sensed through the protein Wsc1 (C. Bermejo and J. Arroyo, unpublished data). Thus, differential regulation of cell wall stress responses seems to be a consequence, at least in part, of how cells are able to sense different types of stress.

An interesting aspect of studying all these mechanisms of fungal adaptation to cell wall stress is the possibility of interfering with them.  $\beta$ -1,3-glucan synthase inhibitors are among the most interesting antifungal compounds recently developed that are applicable to the management of fungal infections. Combined therapies involving the targeting of cell wall biogenesis by inhibiting  $\beta$ -1,3-glucan synthesis together with drugs inhibiting the mechanisms of adaptation to the antifun-

gal drug can be envisioned as a strategy for the development of future successful antifungal therapies.

## Transparency Declaration

All authors declare no conflicts of interests.

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# Global regulators and environmental adaptation in Gram-negative pathogens

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## Abstract

A powerful combination of single-gene studies and whole genome approaches has provided a wealth of information about the regulatory circuits used by bacteria to adapt to the environmental changes that are encountered during infection. The facultative intracellular pathogen *Salmonella enterica* will be used to illustrate how global regulators such as the nucleoid-associated proteins Fis and H-NS collaborate with fluctuations in the superhelicity of the DNA template to modify the gene expression profile of the bacterial cell outside and inside the host.

**Keywords:** DNA supercoiling, *Escherichia coli*, Fis, H-NS, nucleoid-associated protein, *Salmonella enterica*, serovar Typhimurium

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Understanding how bacteria manage their gene expression programmes in response to environmental change is an important goal of modern molecular microbiology. Among the Gram-negative bacteria, the field is perhaps best advanced in *Escherichia coli* K-12, an organism that has been studied at the genetic and physiological levels for many decades [1]. *Salmonella enterica* is a close relative of this model bacterium, and its Typhimurium serovar has been used with great success as a model for bacterial pathogenesis for many years. *S. Typhimurium* offers many of the advantages of *E. coli* as a model organism, but has the additional benefit of being a pathogen whose relationship with its natural host, the mouse, is understood in great detail. In the course of a single round of infection, the bacterium must adapt from an existence in an external environment, such as contaminated food or drink, to the digestive tract of the mammalian host (including the highly acidic environment of the stomach), to an intracellular milieu in the epithelium, to the aggressive environment that characterizes the interior of macrophage, and finally to the deeper tissues of the host. There is also the possibility that the host may shed the bacterium, forcing it to begin the process all over again. From the perspective of research in cellular microbiology, a particularly attractive

aspect of *S. Typhimurium* pathogenesis is its ability to invade and replicate within the cells of its mammalian host [2].

It is clear that *S. Typhimurium* employs a large number of genes to survive and thrive in its relationship with the host. Intensive research efforts have identified scores of virulence genes that are required for one or more steps in the infection process. Some of the most important are grouped together in the bacterial chromosome in so-called pathogenicity islands. Early genetic work had identified some of these genes, but it was the genome sequencing revolution that led to the discovery of the islands. In *S. Typhimurium*, these are large contiguous sections of the chromosome that have DNA with an A + T content that is higher than that of the remainder of the chromosome. This mismatch in DNA sequence composition was part of the reasoning behind the proposal that the islands have been acquired by horizontal gene transfer from a source outside the *Enterobacteriaceae* [3].

The two most important and best-studied *Salmonella* pathogenicity islands are SPII and SPI2. Each of these encodes a distinct type III secretion system and associated effector proteins. SPII is required for host cell invasion, and the effector proteins that it secretes induce bacterial uptake by the non-professional phagocytic cells lining the intestinal lumen [4]. SPI2 is necessary for intracellular survival, and plays an important role in manipulating events within host defense cells, such as the macrophage, in ways that ensure the survival of the bacterium [4]. Other virulence genes are located elsewhere on the *Salmonella* chromosome or on a virulence plasmid that is a characteristic feature of the non-typhoid serovars, of which *S. Typhimurium* is one [3].

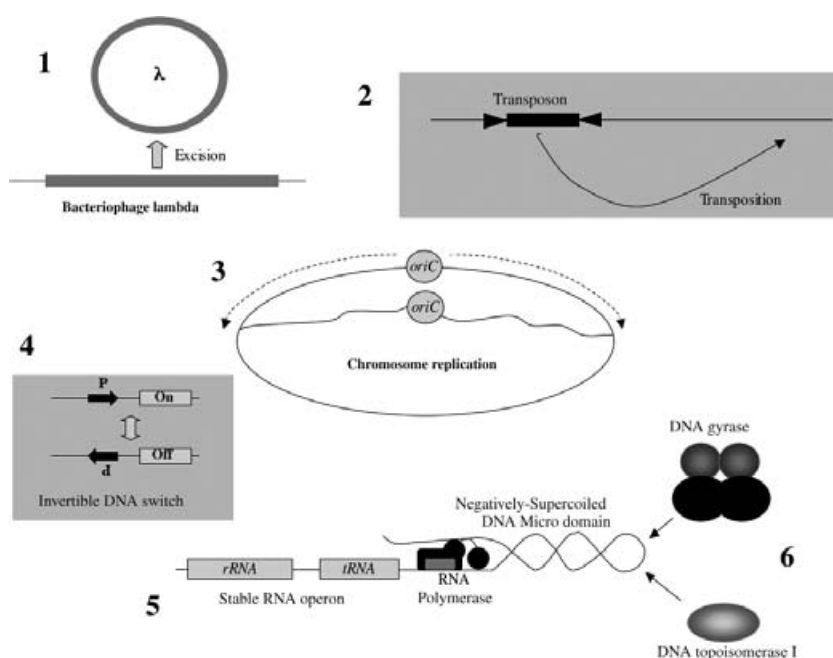


Our understanding of the cell biology of *S. Typhimurium* infection is well advanced. However, a detailed understanding of the regulation of its many virulence genes has lagged behind. In particular, it is still not completely clear how genes that are acquired by lateral transfer mechanisms can become integrated with the existing gene regulatory circuits of the bacterium that receives them, so that they do not compromise the organism's competitive fitness. An attractive hypothesis proposes that a global repressor protein, H-NS, downregulates the horizontally acquired A + T-rich genes, avoiding their inappropriate expression [5–7]. However, this still leaves the problem of how to activate them when they are required. A combination of whole genome and single-gene studies has suggested that the bacterium has evolved a multitude of solutions to this problem.

Transcriptomic investigations using DNA microarrays have identified a striking overlap between the memberships of the H-NS and Fis regulons [5]. Similarly, chromatin immunoprecipitation on chip studies have revealed a remarkable correspondence between the binding sites of the H-NS and Fis proteins in the *E. coli* genome [8]. Fis is the factor for inversion stimulation, an 11.2-kDa DNA-binding protein that was discovered originally as a cofactor in DNA inversion-based genetic switches that are catalysed by members of the serine DNA invertase family of site-specific recombinases. It is now known to have a multitude of roles in the cell; it contributes to transposition, DNA replication, bacteriophage excision, and the control of transcription [9,10] (Fig. 1). In particular, it makes a positive contribution to the transcription of genes

coding for components of the translation machinery. Fis can be either a repressor or an activator of transcription. It binds to an A + T-rich DNA element of degenerate nucleotide sequence, something that is also a feature of H-NS-binding sites. However, H-NS has not been described as activating any promoter directly; its primary direct effect on transcription is one of repression [5].

Fis can also modulate the topology of the genetic material in the cell, especially its degree of negative supercoiling. Most cells, including bacterial cells, maintain their DNA in an underwound state; that is to say, the DNA has a deficit in linking number (the number of times that one strand of the DNA duplex winds around the other). This deficiency places the DNA duplex under torsional stress, enhancing its tendency to form bubbles of unpaired bases that allow a return to a more energetically favourable conformation. This tendency facilitates those transactions of DNA that depend on the formation of locally unpaired bases, such as transcription. Negative supercoiling is introduced to bacterial DNA by the ATP-dependent type II topoisomerase DNA gyrase, and the activity of gyrase is opposed by topoisomerase I, a type I enzyme that removes the negative supercoils [11]. The result is thought to be a homeostatic balance that keeps the degree of negative supercoiling within limits that are appropriate for the biological functions of DNA, including its replication and the expression of the genes contained within it. The Fis protein can influence this process at several levels. It is a transcriptional regulator of the genes encoding DNA gyrase and topoisomerase I, allowing it to influence the supply of these



**Fig. 1.** Regulatory roles of the Fis protein. Fis plays multiple roles in Gram-negative enteric bacteria, including: (1) excision of bacteriophage lambda from the *attλ* site in the *Escherichia coli* chromosome; (2) intramolecular and intermolecular transposition of insertion sequences and transposons; (3) initiation of bidirectional chromosome replication from *oriC*; (4) operation of invertible DNA switches involving site-specific recombination reactions catalysed by serine invertase recombinases; (5) sustained transcription of stable RNA operons through action as a conventional transcription factor and as a topological homeostat; and (6) expression and activity of DNA gyrase and DNA topoisomerase I.



supercoiling-controlling enzymes (Fig. 1). The degenerate nature of the Fis-binding site facilitates the promiscuous interaction of the protein with DNA, and Fis has been shown to bind best to DNA with intermediate levels of DNA supercoiling, which it then helps to preserve in the face of the activities of gyrase and topoisomerase I. The Fis protein can perform this supercoil-preservation function in the vicinity of certain promoters, allowing them to continue functioning after the superhelicity of the remainder of the genome may have become unfavourable for transcription. It should be pointed out that Fis can also act as a conventional transcription factor, influencing RNA polymerase activity at promoters through protein–protein interactions [12].

Mutations in the *fis* gene have pleiotropic effects, as one might expect, given the many systems that it influences. Loss of Fis protein expression results in reduced expression not only of the SPII and SPI2 virulence genes of *S. Typhimurium*, but also the genes involved in motility, many of which contribute to the expression of the third type III secretion system in this species, which is responsible for the deployment and activity of flagella [9]. Fis has also been found to play a role in governing the expression of housekeeping functions that are required for the normal expression of the metabolic pathways that *S. Typhimurium* needs when growing in the gut lumen. Thus, Fis contributes to the management of the gene expression programmes involved in the transition from the gut lumen to an intracellular niche.

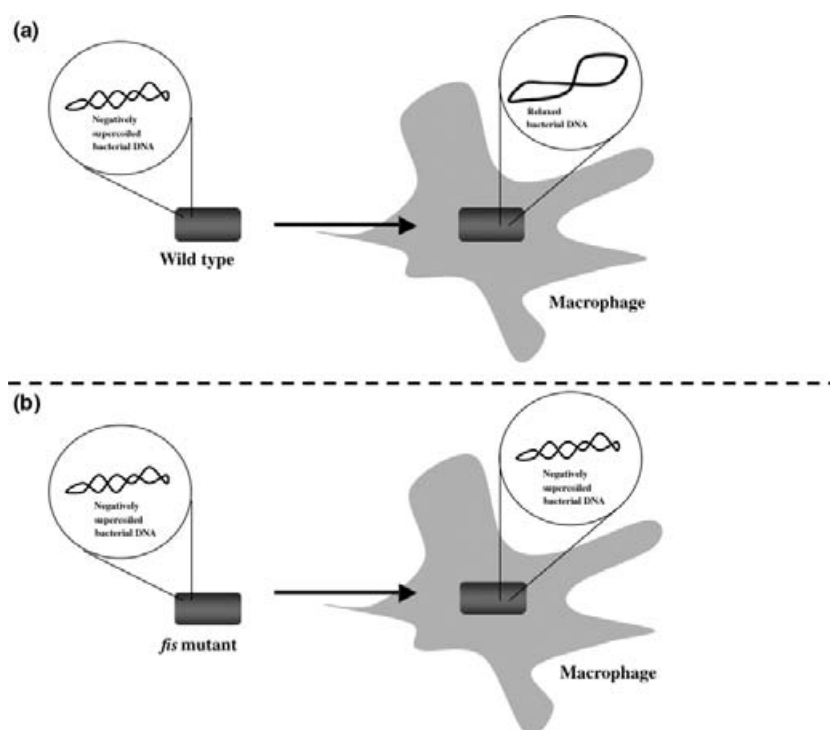
None of these genes displays an absolute requirement for Fis, but by acting to optimize the expression of them all, Fis plays the role of a strategic modulator in these important environmental transitions.

The involvement of the Fis protein in *S. Typhimurium* pathogenicity island gene expression led to the hypothesis that these genes might also respond to changes in negative supercoiling of the DNA. Variations in the degree of DNA supercoiling have been proposed previously as potential global regulators of transcription of both housekeeping and virulence genes [13]. There is ample experimental evidence that the environmental stresses encountered by bacteria during infection can modulate DNA supercoiling, most likely through an alteration of the activity of DNA gyrase as a result of fluctuations in the ratio of ATP and ADP in the cell [11].

Reporter plasmid DNA supercoiling assays have shown that DNA becomes more relaxed in *S. Typhimurium* as the bacteria adapt to life in macrophages [14] (Fig. 2). Furthermore, the Fis protein is required to manage this relaxation process. Interestingly, the SPI2 virulence genes, which are required for survival in the macrophage, have promoters that are stimulated by DNA relaxation, as can be shown by artificially relaxing DNA in bacteria growing in laboratory media by treating the culture with the DNA gyrase-inhibiting drug novobiocin [14].

These observations led to an integrated view of gene regulation in response to environmental stress. In this view,

**Fig. 2.** The effect of adapting to the intracellular environment on the negative supercoiling of DNA in *Salmonella enterica* serovar Typhimurium bacteria. (a) DNA in wild-type bacteria is negatively supercoiled when the microbes are growing outside the macrophage. However, it becomes relaxed soon after engulfment of the bacteria by the host defence cells. (b) A *fis* mutant deficient in the expression of the Fis protein can still be engulfed by macrophage, but its DNA does not show the rapid relaxation that is seen in the wild type. Similarly, the relaxation-dependent genes of the SPI2 pathogenicity island that are required for full adaptation to the macrophage fail to be upregulated, leading to long-term sensitivity of the *fis* mutant bacteria to killing by the host defence cells [14].



crude control operates at the level of the genetic material itself as a result of changes in DNA topology, caused by alterations in the activity of DNA gyrase in combination with the modulating activity of the nucleoid-associated protein Fis. These events occur against a backdrop of transcriptional repression by the H-NS nucleoid-associated protein [5]. More refined control is then imposed by the action of 'conventional' transcription regulators, usually DNA-binding proteins, whose activities are controlled by elements of the dynamic environment. In the case of the pathogenicity islands, these consist of a mixture of proteins encoded by the islands themselves and others that are encoded by genes located in the ancestral genome [15]. These fine-tuning regulators can respond to individual stimuli, such as the presence or absence of specific signalling molecules, allowing fine adjustments to be made to the gene expression programme, optimizing it to suit the complex environment.

## Transparency Declaration

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# Coupled microbial and human systems: evidence for a relationship between infectious disease and gross national product

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## Abstract

We provide evidence that maternal metabolic energy is diverted to increased birth rates in nations experiencing high infectious disease risk. The “economic stoichiometry” of such situations limits the availability and distribution of metabolic resources available for national production. Lowering disease risk, and thus the metabolic energy required for replacement human biomass production, makes energy available for national production during the demographic transition, and increases the national GDP.

**Keywords:** Demographic transition, economic stoichiometry, infectious disease

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## Introduction

The biological flux of materials in the environment is a function of metabolism, establishing for all organisms—ranging from microbes to higher forms—their respective fundamental life history strategies. A metabolic theory of ecology views this flux of materials as a continuum that connects energy flow at many levels, from individual organisms to population and whole ecosystem levels of organization [1,2]. However, as we progress from one scale to the other, new characteristics arise that reveal an increasingly complex, self-organized and power law-dependent network [3,4]. Another feature of complex systems, which is our primary interest here, is the coupling of complex systems between microbial and human systems. Our main thesis is that the metabolic costs of infectious disease incurred by populations/nations results in trade-offs between human biomass production and industrial domestic production.

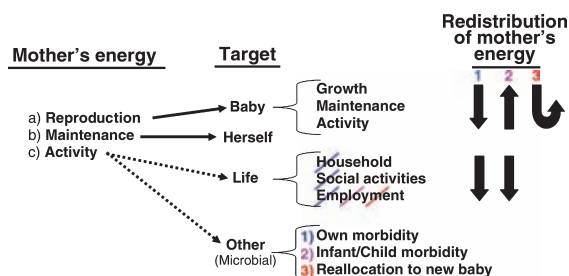
To begin, we recognize that human energy functions are constrained by metabolic rate; our capacity to acquire energy is limited and our ability to redistribute metabolic energy is finite. This constraint operates within the limits of the so-called *energetic-equivalence rule*, which reflects the characteristic connections between individual metabolic rates, rates of

energy flux by populations, and the partitioning of available energy among species within a community [1].

The energy status of an individual is under strict cortical and hypothalamic control [5]. A simple way of putting it is ‘we can only eat so much!’. Appetite is tightly coupled to the metabolic demands of the body, which depends on the energy needed for reproduction, growth, maintenance, physical activity, etc.

However, not only is the energy status of individuals constrained, but so too is it constrained in the life history of the human species. For instance, relative sizes of the human heart, kidney and liver, all of which have relatively high mass-specific metabolic rates, are the expected proportions for a primate of our body size. However, gut and brain size, which also have relatively high mass-specific metabolic rates, are not what we expect. Aiello and Wheeler [6] have shown that, because of a finite metabolic foundation, evolution of the human brain, which is much larger than expected for our body size, was energetically possible because of the evolutionary reduction of the gut. Energy once required to support gut metabolism was given over to support the metabolism of the larger brain. This became possible with the evolution of a higher-quality diet, making less work for the gut to provide the metabolic requirements of our larger brain.

A metabolic growth law for female mammals states that the amount of energy needed for the young female mammal to develop and grow is also that metabolic fraction of energy available to her for reproduction when growth is complete in adult life [7]. The importance of this for human life history in the presence of infectious disease leads to specific expectations concerning the allocations of energy (Fig. 1).



**Fig. 1.** Maternal energy allocations in the presence of infectious disease. See text for explanation of numbering.

Fig. 1 shows that a mother's energy allocations are divided among: (a) that amount of energy she herself took to grow, which, as an adult, she now targets to her baby during reproduction; (b) her basal metabolic rate, or maintenance, which is targeted only to herself; and (c) her daily activities, which are targeted to life, and which are mainly household, social, and employment activities. However, in the presence of infectious disease, most women in the world will expend energy on microbial-related targets, and the redistribution of energy will be expected to ramp away from normal life activities as follows. (1) If she herself is morbidly ill, all household, social and employment activities will be expected to suffer at a level corresponding to the severity of the disease. In this case, energy that she has available for baby and for life will be redistributed downwards; (2) If her child is ill, employment activity is most likely to suffer, this energy now being redistributed to her baby, thus increasing its prospects of recovery and survival; that is, reproductive effort is salvaged if, by increasing parental care, her baby will survive [8], (3) However, Quinlan [8] has shown that when pathogen stress reaches a high and critical threshold, parental care and age at weaning are reduced proportionally to the level of stress. This transfers her energy allocations away from a perceived high-risk endeavour—that is, concentrating energy on a baby increasingly less likely to survive—so that metabolic energy may be re-allocated to those resources needed to produce a new baby. By reducing energy flow to a severely ill child, she increases the probability of regaining positive energy balance, achieving an appropriate pre-pregnancy weight gain, and resuming competent ovarian function [9].

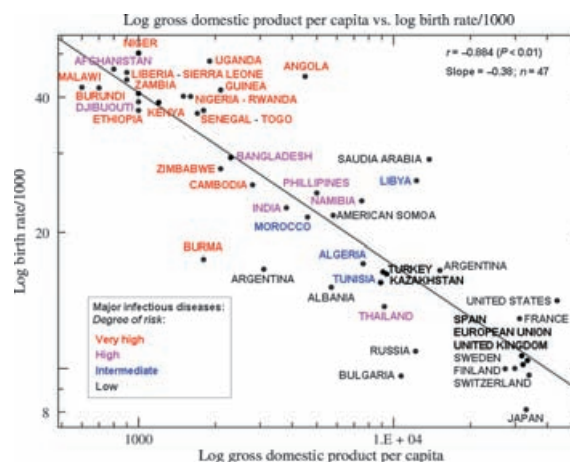
Although human fertility is clearly constrained by individual metabolic flux, it has also been shown that birth rates scale negatively with per capita energy consumption, which includes energy allocations beyond individual biological metabolic requirements, such as the national share of end-consumer use and production [10]. The demographic transition, characterized by increasing energy consumption/production and lowered fertility, is explained by these authors as a function of the cost of raising children, this cost being relatively

low in impoverished nations, resulting in high fertility, and relatively high in wealthy nations, resulting in low fertility. The observed  $-1/3$  power scaling of birth rate is suggested to result from the increased costs of infrastructure and resource distribution to children in industrialized nations.

Because birth rate is directly connected to both infectious diseases and per capita consumption/production, we hypothesize a connection between the metabolic costs incurred by levels of infectious diseases on individuals within nations and their respective per capita share of national production. With confirmation of Moses and Brown's [10] quantitative relationship between birth rate and per capita energy consumption, we provide here the first qualitative evaluation of the role of infectious diseases in causal association with the gross domestic products (GDPs) and demographic profiles of nations.

## Methods

The Central Intelligence Agency World Factbook was consulted for data on crude birth rate (births per 1000 of population per year) and the gross domestic product (GDP) per capita (GDP at purchasing power exchange rates divided by the population) (see <https://www.cia.gov/library/publications/the-world-factbook/index.html>). Data concerning 47 nations are presented in Fig. 2. Superimposed on these data are US government assessments of the degree of risk of major infectious disease risk to government personnel spending less than 3 years in the host country; HIV/AIDS is not included. Every country assessed in the World Factbook for the degree of risk as 'very high', 'high' or 'intermediate' was



**Fig. 2.** Ordinary least-squares regression of log-transformed data on log scales, examining the association between birth rate/1000 and gross domestic product per capita.

included in the analysis, and a random assortment of nations not evaluated for risk were given a rank of 'low' (Fig. 2). It must be noted that these measures of risk probably underestimate the infectious disease load on the local population.

## Results

The results remain true to Moses and Brown's [10] quantitative relationship between national birth rates and per capita energy consumption for 98–166 nations in six periods between 1971 and 1997, whose slopes ranged from  $-0.33$  to  $-0.37$ . The slope observed in our analysis,  $-0.38$ , is slightly higher, which is probably because Moses and Brown [10] subtracted infant mortality from fertility in order to more accurately reflect the number of children raised by parents. However, our interest here lay in the metabolic cost of infectious disease on total fertility, which should include child losses.

As can be qualitatively observed in Fig. 2, nations in which the risk of major infectious disease is very high dominate where relatively high birth rates linearly regress with low GDP per capita. High risk is scattered, although concentrated at relatively lower birth rate and higher GDP per capita than in very high-risk nations. Intermediate-risk nations are located centrally on the regression, and nations in the low-risk disease category dominate where low birth rates linearly regress with high GDP per capita.

## Discussion

We suspect that it is the disease burden on local populations that leads to metabolic costs being diverted to birth rates, these costs and rates increasing together and consequently reducing the net metabolic energy that would otherwise be available for industrial production and national consumption. We believe that this is one factor that limits access of developing nations to the less connected periphery of the network linking global production and export commodities (cf. [11], establishing a connection between the availability and distribution of metabolic resources for nations and a

concept that we dub 'economic stoichiometry'. We further suspect that the classical demographic transition is a result of increased access to healthcare and reductions in the risk of infectious disease. Lowering risks allows women to allocate reproductive metabolic energy to fewer surviving children and a larger share of metabolic energy to various activities, particularly employment.

We have shown that if a nation's risk of infectious disease is low, the cumulative metabolic energy otherwise required for replacement human biomass production is probably converted into national production, effectively driving the demographic transition and increasing the national GDP.

## Transparency Declaration

The author declares no conflicts of interest.

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# Tracking bacterial responses to global warming with an ecotype-based systematics

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## Abstract

The broadly defined species of bacterial systematics frequently contain unnamed and unrecognized populations (ecotypes) differing in physiology, genome content, and ecology. Without formal recognition of such ecotypes, it is difficult for microbial ecologists to detect replacement of one ecotype by another in the face of global warming. The ecotype simulation algorithm has proved capable of supporting investigation of such replacements, as it has detected temperature-distinguished ecotypes that are invisible to the present bacterial systematics. Creating an ecotype-based systematics will help to identify the units of diversity that we will want to track as we seek to observe the early microbial responses to global warming.

**Keywords:** *Bacillus*, ecology, periodic selection, speciation, species concept

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## Introduction

Thermometers and shrinking ice packs provide the physical alarms that the world's climate is warming, but biotic changes offer the most visceral harbingers of a hot future world—polar bears adrift on shrinking ice on an open Arctic Ocean, tropical birds appearing on the checklists of temperate birders, and European sheep succumbing to an African disease. As animals, plants and microbes move polewards, ecologists are challenged to track the responses of organisms to global warming. These efforts should help our species to accommodate to the biotic challenges of a warmer world. More generally, global warming presents a novel opportunity for microbial ecologists to investigate the roles of migration, adaptation and speciation in accommodating environmental changes.

Zoologists and botanists have already made significant progress in tracking the responses of many individual animal and plant species to global warming, e.g. showing how various marine fish have moved northwards in recent decades [1]. In addition, zoologists and botanists have predicted the future geographical responses of individual species. For example, the very closely related oak species *Quercus*

*douglasii* (blue oak) and *Quercus lobata* (valley oak) of California, with slightly different habitat requirements, have each been predicted to contract from warmer habitats and to expand into adjacent cooler habitats [2]. For our purposes, this case is particularly interesting, because the more drought-tolerant blue oak is invading the present habitat of the more mesophilic valley oak. Such a prediction is possible because, like most pairs of closely related plant and animal species, these oak species are narrowly defined so that each species is homogeneous within itself (at least at any one location), and distinct from the other in its physiology and preferred microhabitats [3,4]. Thus, the finely tuned systematics of plants (and animals) allows us to observe and predict the replacement of one extremely close relative by another in the face of global change.

We can imagine how difficult it would be to track or predict geographical range changes in oaks if plant systematics did not identify all of the closely related, ecologically distinct oak species within a region. Suppose instead that the only recognized oak taxon was the genus *Quercus* (i.e. with no individual species recognized), and that it was our job to track responses to global change in the genus *Quercus* at large. Changing our focus from the individual species to the *Quercus* amalgam, containing blue oak and valley oak, as well as dozens of other oak species from this region, would blind us to replacements of one subgroup by another. Unfortunately, this is exactly the situation that bacterial systematics leaves us in. The named, recognized species of bacterial systematics are defined quite broadly, much like a genus of animals or plants, such that the typical bacterial species is



extremely diverse in its physiology and genome content, but most fundamentally in its ecology [5,6].

The broad brush of bacterial systematics is seen clearly in the case of *Escherichia coli* [7]. Three strains within this species, one non-pathogenic, one uropathogenic, and the other enterohaemorrhagic, have been shown to share only 39% of their genes. Most significant, however, is the fact that these strains, so profoundly distinct in their ecology and clearly long divergent (having diverged so much in genome content), are judged by bacterial systematics to reside within the same species taxon. Given the wide vision of bacterial systematics for inclusion of diverse populations within a species, one can imagine that tracking or predicting the geographical responses of unnamed, unrecognized, ecologically distinct populations within a 'species' would be challenging, indeed.

We have recently proposed a paradigm shift in bacterial systematics that aims to incorporate ecological diversification into analysis of bacterial diversity [5,8]. Here, I will argue that this 'ecotype'-based systematics will enable microbiologists to observe geographical responses to global warming that would be invisible to the current systematics of bacteria. To this end, I will demonstrate that the new systematics can identify extremely closely related bacterial populations, with different temperature adaptations, that would be unrecognized by the prevailing systematics.

### The rationale for sequence-based demarcation of ecotypes

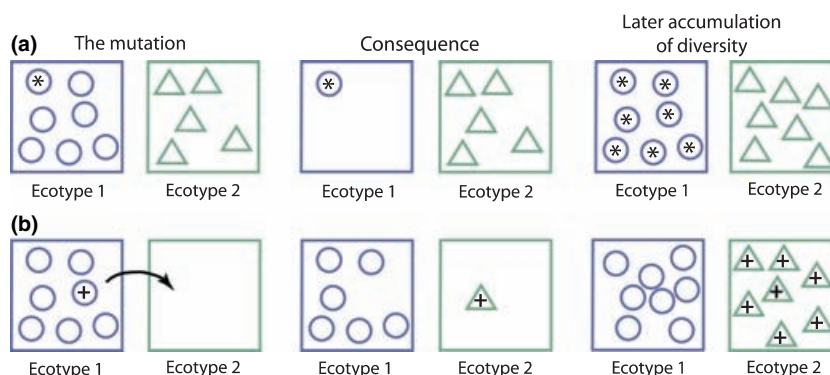
Identification of very closely related, ecologically distinct populations is more difficult in bacteriology than in zoology or botany [5]. Zoologists can anticipate, for a given animal group, the traits that determine the ecological niche. For example, a zoologist studying songbirds expects that the various species will differ in their bill shapes and sizes (which determine the kind and size of food consumed), and so can easily identify all the ecologically distinct populations within a community. However, a bacteriologist cannot anticipate with confidence the characteristics determining niche differences between closest relatives, even for a well-studied taxon. This is because the creation of new populations is frequently brought about through horizontal genetic transfer, where a new enzyme function or pathway is introduced into a recipient organism from any of a large set of potential donor organisms [5,9]. As we do not know what aspects of physiology to focus on, discovery of ecological diversity among closely related bacteria cannot depend on physiological analysis alone. Fortunately, DNA sequence surveys are well suited to discovering ecologically distinct bacterial populations ('ecotypes') [5].

We have defined an ecotype as a clade of bacteria that are ecologically similar to one another, such that genetic diversity within the ecotype is limited by a cohesive force, either periodic selection or genetic drift, or both (Fig. 1) [5]. In this model, diversity within an ecotype is ephemeral, persisting only until the next periodic selection event, when diversity is brought to near zero at all loci, or until purged by genetic drift. Divergence becomes permanent when a mutation (or recombination event) places the organism into a new ecological niche and thereby founds a new ecotype. Because the new ecotype is ecologically distinct from the parental ecotype, periodic selection events in the parental ecotype cannot extinguish the founding organism and its descendants (Fig. 1). The new ecotype thus escapes the periodic selection events of the parental ecotype, and the two new ecotypes are free to diverge indefinitely. Ecotypes defined in this way bear the characteristics attributed to species by biologists outside of microbiology: each ecotype is cohesive (with diversity constrained by periodic selection and/or drift), different ecotypes may diverge indefinitely from one another, and the different ecotypes are ecologically distinct [10].

Such ecotypes may be revealed through molecular surveys, provided that one particular model of bacterial evolution holds. This is the stable ecotype model, where each ecotype persists through a long history of recurrent periodic selection events, and new ecotypes are only rarely formed. In this model, each ecotype is ideally expected to correspond to a separate DNA sequence cluster, for any gene shared among ecotypes, as periodic selection purges diversity at all loci, within but not between ecotypes, leading to a steady accrual of sequence divergence between ecotypes (Fig. 1).

### The ecotype simulation approach

Most sequence-based bacterial phylogenies are complex, with many levels of clusters and subclusters within clusters (Fig. 2), and so it is generally not clear which level of sequence cluster should correspond to ecotypes. Bacterial systematics has utilized various universal molecular criteria for demarcating clusters expected to be of biological significance. For example, species have been demarcated for decades under the guidance of a universal criterion of genome content similarity, as quantified by DNA-DNA hybridization [11]. More recently, species demarcation has been guided by divergence at the 16S rRNA locus, first with a 3% cut-off and more recently with a 1% cut-off [12]. However, there is no theoretical rationale for these cut-offs to correspond to biologically significant clades with species-like properties, and



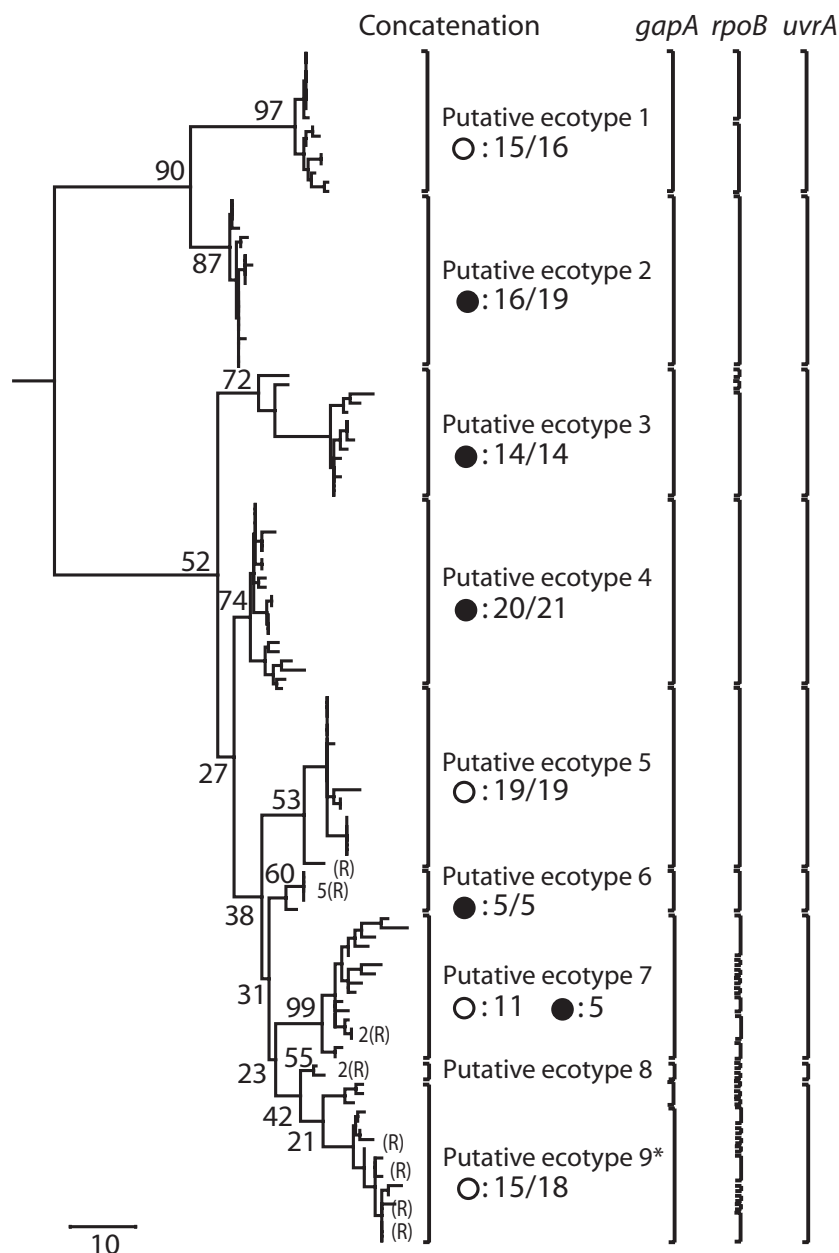
**Fig. 1.** Two classes of mutation and recombination events that determine ecotype diversity in bacteria. Circles and triangles represent different genotypes within ecotypes 1 and 2, respectively; asterisks represent adaptive mutations and crosses represent ecotype formation mutations. (a) Periodic selection mutations. These improve the fitness of an individual such that the mutant and its descendants (ecotype) outcompete all other cells within the ecological niche (i.e. cells of the same ecotype); these mutations do not affect the diversity within other ecotypes, because ecological differences prevent direct competition. Periodic selection leads to the distinctness of ecotypes by purging the divergence within but not between ecotypes. (b) Ecotype formation mutations. Here a mutation or recombination event allows the cell to occupy a new ecological niche, founding a new ecotype. The ecotype formation mutant, as well as its descendants, can no longer be extinguished by periodic selection events from its former ecotype [17]. (Used with permission from Landes Publishers.)

nor is it clear that any particular cut-off should apply to all bacteria [5]. In any case, applying the cut-offs embraced by systematists has led to the enormous ecological and physiological diversity seen within *E. coli* and within many other species.

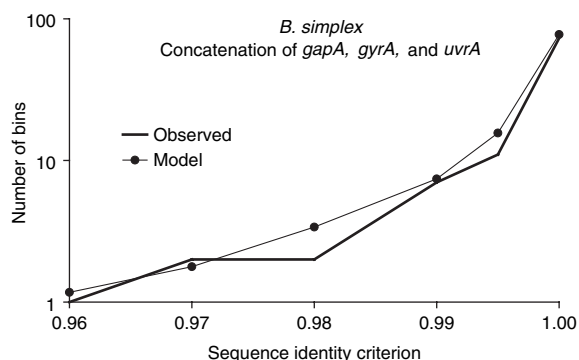
We have proposed a theory-based approach called ecotype simulation to derive cut-offs that are appropriate for demarcating a particular clade's ecotypes, allowing that different bacterial groups may have different cut-offs [8]. The ecotype simulation approach begins by characterizing the sequence diversity within a clade as the number of sequence clusters (or bins) present for different sequence identity criteria (Fig. 3) [13,14]. The number of sequence clusters at a particular sequence identity level represents the number of lineages at some point in the past that have survived to the present; thus, the sequence diversity curve represents the history of splitting of lineages within the clade [14]. The ecotype simulation algorithm estimates the rates of periodic selection and drift, the net rate of ecotype formation (taking into account ecotype extinction), and the number of ecotypes ( $n$ ), so as to yield a clade's sequence diversity pattern (Fig. 3) with maximum likelihood. Individual ecotypes are demarcated by determining the largest subclades that are each consistent with containing a single ecotype (i.e. such that  $n = 1$  for the subclade). Further details of ecotype simulation may be found in our previous work [8], and the software may be downloaded from <http://fcohan.web.wesleyan.edu/ecosim/>.

We have applied ecotype simulation to analyse 131 strains of *Bacillus simplex* isolated from two 'Evolution Canyons' of

Israel [8]. Each 'Evolution Canyon' is an arid canyon running east to west, providing two major habitats differing in solar insolation—these are the north-facing slope (NFS) and the south-facing slope (SFS). Ecotype simulation analysis inferred nine putative ecotypes within *B. simplex* (Fig. 2). We were able to confirm that many of these groups were ecologically distinct, first by comparing the putative ecotypes for their associations with the two major habitats. For example, at the top of Fig. 2 is a clade containing putative ecotypes 1 and 2, which were distinguished from one another by their strong associations with the SFS and NSF, respectively; other putative ecotypes in other clades were also distinguished by their associations with the NSF and SFS [8]. The ecological distinctness of the putative ecotypes was also corroborated by physiological differences. The SFS-associated ecotypes contained greater levels of high-temperature-adapting isomethyl-branched fatty acids than the NFS-associated ecotypes [15]. Also, the SFS ecotypes have shown higher growth rates than NFS ecotypes at a stressfully high temperature, whereas the differences disappeared at optimal temperatures. The NFS-adapted and SFS-adapted ecotypes were not different in their sensitivities to UV-C radiation, so temperature appears to be an important component of divergence between these ecotypes. We have not yet investigated other possible differences in niche among ecotypes, such as differences in organic resources utilized, or differences in interactions with other microbes. Other differences yet to be discovered may explain the coexistence of multiple ecotypes on the same slope, e.g. putative ecotypes 1, 5, 7 and 9 on the SFS.



**Fig. 2.** Phylogeny and ecotype demarcation of *Bacillus simplex* from the 'Evolution Canyons' of Israel. The phylogeny is based on a concatenation of three protein-coding genes, with recombinant sequences removed. The phylogeny contains many clusters and subclusters within clusters, and it is not intuitively clear how to demarcate the ecologically significant groups without a theory. Using ecotype simulation, ecotypes were demarcated as the most inclusive clades that were each consistent with being a single ecotype. Ecotype demarcations are indicated by brackets, as based on analysis of the concatenation as well as each individual gene. The ecotype demarcations were similar as based on the concatenation and the individual genes, except that the more rapidly evolving gene *rpoB* tended to split the ecotypes determined by analysis of the concatenation. A group of related recombinants is indicated by 'R' following the number of recombinants. For isolates that had recombined at one gene locus, ecotype placement was determined by ecotype simulation of the two genes that had not recombined. With one exception, demarcated ecotypes were supported as monophyletic groups in at least 50% of bootstrap replications (percentage bootstrap support indicated at nodes); the exception is asterisked to indicate that its phylogenetic status is tentative, pending additional sequence data. Microhabitat sources were the south-facing slope (○) and the north-facing slope (●). For each ecotype represented by at least four isolates, the principal microhabitat source(s) is indicated. If one microhabitat provided at least 80% of the isolates, the principal microhabitat source is indicated; for ecotypes not so dominated by a single source, all microhabitat sources are indicated. Note that the prevailing practice of bacterial systematics has included all of this diversity within one species [8]. (Used with permission from the National Academy of Sciences.)



**Fig. 3.** Observed and modelled clade sequence diversity patterns. Sequences for a gene (or a concatenation of genes) were binned into clusters with different levels of minimum pairwise identity. The curves represent the diversity among 116 *Bacillus simplex* isolates from 'Evolution Canyons' I and II, as based on a concatenation of *gapA*, *rpoB*, and *uvrA*, with 15 recombinant organisms removed [8]. The individual points for the model curve are means based on 1000 replications of the maximum-likelihood solution. (Used with permission from the National Academy of Sciences.)

All of the *B. simplex* ecotypes from the 'Evolution Canyons' are extremely closely related to one another, as revealed by complete identity in their 16S rRNA sequences [8]. Thus, in all likelihood, these ecotypes would remain unrecognized and unnamed with the prevailing practice of bacterial systematics [8].

We have also applied ecotype simulation to a clade of hot spring cyanobacteria from Yellowstone National Park in the USA [16]. Within the A and A' subclades of *Synechococcus*, ecotype simulation identified putative ecotypes that were confirmed to be ecologically distinct by differences in associations with temperature and depth in the photic zone. Some of these confirmed ecotypes were only 0.7% divergent at 16S rRNA, and so would probably be unrecognized by bacterial systematics.

## Towards an ecotype-based systematics

Ecotype simulation promises to identify the extremely closely related ecotypes that have up to now been included within the recognized species of bacterial systematics. We have previously proposed a protocol for incorporating ecotypes and ecological diversification into bacterial systematics, taking into account that factors other than periodic selection may contribute to sequence clustering in certain lineages [5,8]. The first step is to infer putative ecotypes through ecotype simulation analysis of DNA sequence data (or through another theory-based model of bacterial evolution and

ecology). The second step is to confirm that the putative ecotypes so identified are actually distinct in their ecology in nature. This could involve comparison of microgeographical distribution, physiology, genome content and/or genome-wide gene expression among putative ecotypes. We have suggested that ecotypes discovered within the phylogenetic range of an existing, named species (e.g. within 1% divergence at 16S rRNA) should be named as a trinomial 'ecovar' within the established species; also, newly discovered ecotypes that are outside the phylogenetic range of existing species should each be named as a separate species [5].

Recognition of ecotypes will yield the systematic infrastructure with which to discern early and subtle responses to global warming. Just as botanists can predict or track expansion of the drought-tolerant blue oak into the cooler habitats now held by the valley oak, the ecotype-based systematics will allow microbial ecologists to track replacement of a mesophilic ecotype by an extremely close relative adapted to hotter microclimates. Ecotype simulation has proved capable of supporting such observations in the future, as it has detected temperature-distinguished ecotypes that are invisible to the present systematics, in the cases of *Bacillus* and *Synechococcus* [8,16]. I expect that many broadly defined species recognized by systematics will be shown to contain temperature-distinguished ecotypes as well. These are the units of diversity that we will want to track as we seek to observe the early microbial responses to global warming.

## Transparency Declaration

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# Impact of space, time and complex environments on microbial communities

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## Abstract

The unparalleled accumulation of biological and contextual data is currently revolutionizing the way environmental microbiologists address ecological questions. Here, we briefly review the likely causes that may explain this remarkable scientific revolution and present a synthesized view about how to describe microbial communities in their complex environmental context.

**Keywords:** Ecology, diversity, modelling, theory, multivariate

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Environmental microbiology is currently undergoing a revolution owing to the increasing accumulation of biological information and of contextual environmental parameters [1–4]. This new era may well be described as a microbial ecologist's dream. Key ecological questions such as the extent of variation of microbial diversity and abundance with respect to spatial, temporal or environmental parameters may start to be addressed. Here, we briefly review the likely causes for this dramatic advance in discovery and present a synthesized view about how to describe microbial communities in their complex environmental context. Examples from our own work are presented to illustrate possible combinations of methods, strategies and approaches that could lead to new discoveries.

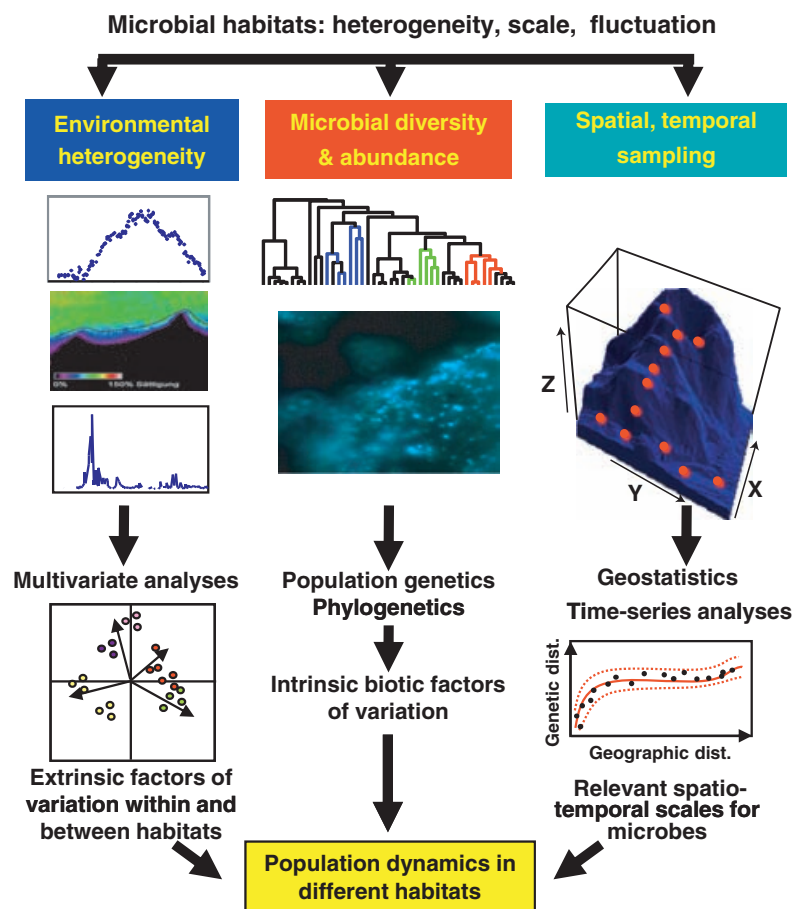
Microbial communities thriving in different habitats in soil or marine environments may be studied via a combination of high-throughput molecular techniques and powerful multivariate analyses (Fig. 1). Microbial diversity can be routinely inferred using variations in molecular markers (e.g. 16S rRNA genes, protein-coding genes) and phenotypic characteristics. The abundance of organisms can be quantified using plate counting (for the culturable fractions) or by microscopic observations (e.g. fluorescent *in situ* hybridization). The diversity among and between communities and populations can thus be determined using phylogenetic reconstruction. Environmental heterogeneity can be measured by collecting contextual information about the microbial habitat

from which the organisms were isolated. Because most microbial ecology studies are of an exploratory nature (i.e. one cannot tell *a priori* what the main environmental factors are), multivariate analyses may be used to reduce the dimensionality of those additional datasets [3]. In natural ecosystems, complexity and multidimensionality are common features: multiple factors, variables and parameters are generally collected and stored in specialized databases. There is thus a need to quantify the amount of biological variation that can be explained by environmental, temporal and spatial factors in order to establish the strength of our current understanding and predictive abilities about changes in the microbial world. This ecosystem approach may shed light on the predictability of natural processes governed by microbial populations. A summary of the overall strategy that combines microbial data with environmental, spatial and temporal parameters is presented (Fig. 1).

The current revolution in microbial ecology is taking place because the primary materials (i.e. DNA and protein sequences) that microbiologists use to study large-scale diversity patterns are being accumulated at an exponential rate, and because of new molecular techniques (e.g. pyrosequencing, whole genome amplification, and metagenomics), which are speeding up the process even further [5]. In-depth estimation of microbial diversity will probably be soon complemented by assessing numerous samples in parallel, a process that is so far rather limited. It can be assumed that in the near future a full understanding of the depth and breadth of microbial diversity will be possible, thus providing new insights into the central role of microbes on earth.

Refinement of existing molecular techniques may also lead to significant improvements in the description of diversity patterns. For instance, in multilocus sequence typing





**Fig. 1.** Synthetic scheme to combine microbial diversity and abundance with environmental, spatial and temporal parameters in a coherent framework.

approaches, seven to ten loci are typically used to compare evolution across microbial strains. We showed that not all markers yielded meaningful results when compared with the phylogenetic information deduced from genomic data [6]. A more accurate phylogeny of the *Escherichia coli* group was obtained on the basis of just three genes, in comparison with the concatenated alignment of eight genes that are commonly employed for phylogenetic purposes. Those results were reproducible within *Salmonella*, *Burkholderia* and *Shewanella*, suggesting a broad applicability for those analyses.

Despite huge progress in obtaining sequence information, advances in the isolation and cultivation of microbes remain central to a better understanding of the coupling of diversity, function and metabolic potential of specific strains from the environment. For instance, the diversity of *Burkholderia cepacia* isolates was investigated at the community, species and intraspecific level by using a combination of molecular and culture-dependent techniques [7]. Efficient strategies to detect, isolate and screen large numbers of *B. cepacia* isolates from soil and rhizosphere samples were used to offer a fast and reliable diagnostic assay for members of this bacterial complex that colonize samples as different as soil, water,

sediment, or human lungs. The combination of molecular data with advanced ecological modelling tools also proved valuable. Variations in community composition, abundance and diversity were determined as functions of environmental and spatial parameters for various rhizosphere samples [8]. A powerful spatial modelling approach was used to examine all spatial scales that significantly structured the microbial community and to relate spatial scales with environmental heterogeneity [9]. It was then possible to determine the amount of biological variation that could be explained by different spatial scales and environmental fluctuations of contextual physicochemical parameters at different taxonomic levels. Interestingly, the intraspecific diversity showed significant variation as a function of nearly all scales present in the sampling strategy. This may indicate very complex structuring of both space (i.e. historical factors) and contemporary environmental parameters at the genotype level in microbial populations [8]. Microbial ecologists have traditionally favoured the environmental control over the historical (spatial distance) hypothesis to explain microbial patterns in the environment [1,4,8]. Determining the effects of spatial structure in biological data is a recurring topic in traditional

landscape ecology, and microbiologists should take advantage of the numerous existing examples to apply those approaches to their own studies. Statistical tools, in this respect, may be of great help in separating the respective effects of pure spatial and pure environmental variation [3]. Also, their covariation, i.e. how much spatial structuring indirectly affects biotic variables through environmental structuring, can be quantified [10].

Marine coastal sediments are examples of highly productive ecosystems that play important roles in carbon and nitrogen recycling, but whose microbial communities are still poorly understood. Not only do sediments cover a large area of the earth's surface, but they are also highly colonized by microbial communities. We recently applied the 454 massive parallel tag sequencing technique to sandy sediments from the North Sea island Sylt through a collaboration with the International Census of Marine Microbes (ICoMM; <http://icomm.mbl.edu/>). The objectives were to obtain a basic understanding of the depth of the microbial diversity in coastal sediments and to identify the main environmental drivers that may structure sand-associated communities. The 454 massively parallel tag sequencing strategy has been shown to be able to detect the rare biosphere that generally escapes traditional molecular approaches [11]. Multivariate analyses, when applied to massive parallel tag sequencing-generated data, could extract the main amounts of variation from highly dimensional tables, despite the huge dataset size of more than 160 000 sequence tags. The results suggest that a broad range of sequence tags are present in Sylt sands, consistent with an extensive diversity and high turnover of community diversity in such dynamic ecosystems (A Gobet, A Ramette, ML Sogin and A Boetius, unpublished).

Although the tendency to work with larger datasets is becoming obvious, a pitfall would be to blindly embark on generating more data without clear objectives. The accumulation of data should ideally be accompanied by an accumulation of knowledge. Several authors have already warned us against this lack of strategy [2]. The development of sound theories that are falsifiable is needed to provide the necessary scheme to advance science into a coherent framework. To reach this goal, environmental microbiologists have to face the big challenge of understanding complexity and diversity by constantly updating their approaches, theories and methods.

In conclusion, it is now possible to assess the impact of space, time and complex environments on microbial communities and to quantify interactions among factors. This new possibility has been made possible by a conjoint revolution in data acquisition, storage and processing. The next challenge may be the generation of microbial diversity theories that will allow further comparisons with those of macro-organisms or that can be tested across various ecosystems. For instance, recent developments in the study of microbial biogeography [1,4,12] may be seen as a prelude to a more dramatic revolution in the understanding of changes in microbial communities in complex environments.

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## Transparency Declaration

All authors declare no conflicts of interests.

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# Recombinant bacteria for environmental release: what went wrong and what we have learnt from it

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## Abstract

From a biotechnological point of view, bacteria can be seen as either pathogens to target with new drugs or as biocatalysts for large-scale processes in industry, agriculture or the environment. The last includes the exploitation of bacterial activities for bioremediation of toxic waste either *in situ* or *ex situ*. The onset of genetic engineering in the late 70s opened the possibility of tailoring recombinant bacteria for environmental release, aimed at biodegradation of otherwise recalcitrant chemicals. However, a few decades later the outcome of this prospect has been quite meager. The literature counts very few cases where the use of genetically engineered bacteria has been proven to be more efficient than natural microorganisms in elimination of recalcitrant compounds under natural (not laboratory) conditions. Fortunately, the emergence of Systems and Synthetic Biology in the last few years is helping to identify what were the caveats of the former approaches and how to correct them. In addition, robust design concepts imported from process engineering provides fresh approaches to the challenge of designing microorganisms *à la carte* for environmental applications.

**Keywords:** Biodegradation, *Pseudomonas*, recombinant antibodies, synthetic biology

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Since the early 1980s, genetic engineering of soil bacteria has been claimed to have an extraordinary potential for remediation of environmental pollution, as long as it ultimately produced the design of superior contaminant-breaking live microbial catalysts [1,2]. However, despite intensive efforts in Europe and the USA, the success of such approaches has been very limited thus far [3]. Many problems have been encountered in constructing strains that perform well not only in the laboratory, but also under real environmental conditions (Table 1). It is intriguing that metabolic engineering, which is at the core of any refactored or improved biodegradative pathway, is not the main problem. Rather, bacteria engineered for bioremediation, biocatalysis, or biosensing, require the adoption of hosts, genetic tools and even conceptual frames that diverge from those used for laboratory microorganisms in laboratory-based experiments. In the environment, new information borne by implanted genes and genetic circuits must be stably inherited in the absence of selective pressure, must not be associated with antibiotics, and must not cause the loss of ecological fitness in the carrier.

Because of their genetic promiscuity, the Tn5 and Tn7 transposition systems are optimal sources of biological

modules that can be claimed to be authentically context-independent. Thus, they are attractive for developing dedicated molecular tools. We have constructed a large collection of mini-transposon vectors based on Tn5 [4] and Tn7 that allow stable integration of multiple DNA segments into the chromosomes of a whole range of robust Gram-negative soil bacteria such as *Pseudomonas putida*. These vectors have been instrumental in designing strains that are able to aerobically degrade the otherwise recalcitrant compound 2Cl-toluene in soil [5]. To this end, we inserted catabolic segments with the toluene dioxygenase of the TOD system of *P. putida* FI (*todC1C2BA*) and the entire *upper* TOL pathway from the pVW0 plasmid of *P. putida* mt-2 into the chromosome of one 2-chlorobenzoate-degrading *Pseudomonas* strain. The resulting cells possessed not only the inserted genetic information, but also the functional ability to mineralize 2-chlorotoluene.

However, although these strains did convert the substrate into 2-chlorobenzoate, they failed to grow with 2Cl-toluene as the only carbon source and produced undesirable dead-end hydroxylated products. These results indicate that the real bottlenecks in engineering the degradation of certain pollutants do not rest solely on the enzymology of the process. The rise of systems biology and *omics* technologies has shed some light on why biotransformations that should work well from an enzymatic point of view happen not to operate properly in the wider context of a live cell. Some revealing pieces of information have been recently published [6,7]

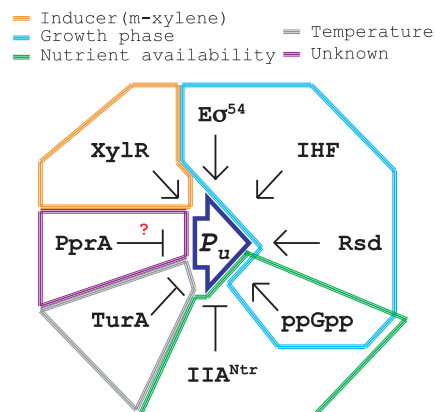
**Table 1. Challenges in constructing genetically engineered bacteria intended for environmental release**

Problem	Solution
<i>Escherichia coli</i> laboratory strains not robust enough	Use environmental soil bacteria
Antibiotic resistance as selection markers	Non-antibiotic markers and excisable resistance
Plasmids as carriers of engineered traits	Stable chromosomal integration
Expression dependent on chemical inducers	Expression dependent on environmental inputs
Strong selection against implanted genes/circuits	Orthogonalization of engineered functions

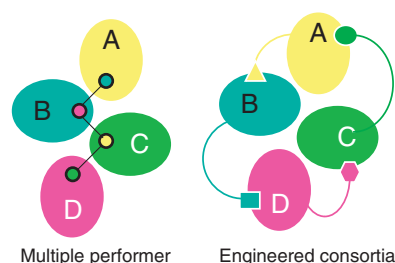
suggesting that bacteria exposed to aromatic chemicals (toluene and the like) redirect the transcriptional machinery to activate stress response genes. This helps cells to cope with the exposure to organic solvents but thus diverts RNA polymerase from the desired task of expressing catabolic genes for toluene biodegradation.

A separate bottleneck relates to the use of heterologous expression systems for achieving production of the desired enzymes in host cells. Most naturally occurring promoters, even the simplest, have more than two input functions. In engineered biological systems intended to perform in the field, this certainty cannot be overlooked. Let us take, for instance, the *Pu* promoter encoded in the pWW0 TOL plasmid for biodegradation of this aromatic hydrocarbon in *P. putida* mt-2 [8]. *Pu* belongs to the class of promoters that depend on the alternative sigma factor  $\sigma^{54}$  and is activated at a distance by XylR, an *m*-xylene-responsive activator (Fig. 1). XylR can be mutated to respond to non-natural aromatic effectors [9,10], making it an ideal basis for developing a large number of aromatic-inducible expression systems ideal for engineering transcriptional circuits. However, the action *in vivo* of XylR and *m*-xylene on *Pu* depends not only on these two inputs but also on a plethora of additional factors and signals that tune promoter output to the general physiological and metabolic conditions of the cells [8]. To varying degrees, many other natural promoters are affected by the same problem, as prokaryotic regulatory economy tends to compress control elements in increasingly shorter DNA sequences [11]. Fortunately, it is possible to avoid such compressions and come up with promoters and genes relieved of such physiological control [11,12], causing a robust inducer-dependent expression.

A final factor that limits genetic engineering of environmental catalysis is the realization that many, perhaps most, intracellular polypeptides associate into multiprotein structures [13,14] in which many products of orphan genes are likely to scaffold enzymatic complexes [13,15,16]. Furthermore, there are indications that functionally related gene clusters or genomic islands are located in distinct places of



**Fig. 1.** Factors that affect expression *in vivo* of the sigma-54-dependent promoter *Pu* of the TOL plasmid pWW0 of *m*-xylene-degrading *Pseudomonas putida* mt-2. *Pu* can be transcribed *in vitro* by combining purified IHf, the sigma factor core RNAP and activated XylR. However, the same promoter is subject *in vivo* to a plethora of additional factors and inputs, which influence to various degrees the activity of *Pu* under diverse growth or stress conditions. Mechanistically, such signals enter through the integration host factor (IHf), the IIA<sup>Ntr</sup> protein, sigma factor competition, ppGpp levels, temperature, the TurA histone-like proteins, and perhaps other additional inputs.



**Fig. 2.** Engineering bacterial consortia with adhesins *à la carte*. Bacteria A, B and C, which are unable to degrade a given chemical, may, however, bear genes encoding enzymes which, when put together, can give rise to a novel metabolic pathway. However, such bacteria may not naturally have any tendency to associate with each other (right). However, consortia can be forced to form by expressing on the surface of the cells specific adhesins (e.g. single-chain antibodies) anchored to the cell envelope with autotransporter domains (right; see text for explanation).

the chromosome that target their transcription to given spots of the cytoplasm [17]. This means that each protein needs to be expressed and located in an intracellular physical niche to optimally perform its function. Polypeptides unable to fit within such assemblies might be rendered non-functional and eventually rejected through a simple Darwinian mechanism. Although inserting extra DNA into a cell is straightforward, implantation of the encoded proteins in the molecular ecosystem of the bacterial cytoplasm might be severely counterselected [18].

Do these numerous constraints in designing bacteria intended for environmental release mean the end of this scientific and biotechnological field? Fortunately, the situation is changing rapidly with the advent of synthetic biology and its emphasis on robust design concepts, orthogonality, i.e. context independence, and definition of systems boundaries. In fact, engineering bacteria for bioremediation, biocatalysis or biosensing is receiving renewed attention in view of the possibilities opened by such an emerging discipline [19,20].

Apart from single-strain manipulation, environmental applications of engineered bacteria also encompass the assembly of microbial communities deliberately structured to combine qualities possessed separately by different bacterial strains (Fig. 2). Accordingly, we have devised a general genetic method for surface display of artificial adhesins on the surface of Gram-negative bacteria [21]. This is based on the so-called autotransporter secretion systems, which export and attach both small peptides and completely folded and active proteins to the exterior of the cells [22]. Such a system has been employed for engineering *Ralstonia eutropha* cells coated with a rat metallothionein, aimed at bioadsorption of heavy metals in soil, which is displayed on the cell surface as a metallothionein-autotransporter hybrid [23]. Finally, the same surface-anchor procedure was employed for targeting expression of Fos/Jun protein dimerization domains to the surface of *Escherichia coli* [21] and for coating live cells with recombinant camel antibodies [22]. All of these new developments herald what can be seen as a rebirth of genetic engineering for environmental applications.

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## Transparency Declaration

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# Fitness cost of drug resistance in *Mycobacterium tuberculosis*

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## Abstract

Multidrug-resistant (MDR) - and extensively drug-resistant (XDR) - forms of tuberculosis are growing public health problems. Mathematical models predict that the future of the MDR and XDR tuberculosis epidemics depends in part on the competitive fitness of drug-resistant strains. Here, recent experimental and molecular epidemiological data that illustrate how heterogeneity among drug-resistant strains of *Mycobacterium tuberculosis* can influence the relative fitness and transmission of this pathogen are reviewed.

**Keywords:** Antibiotic, competition, genotype, mutation, mycobacteria, transmission

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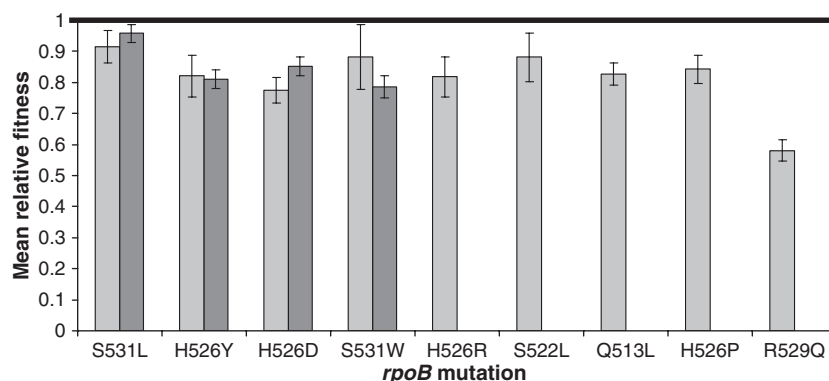
Drug resistance has become a problem of global proportions, with major consequences for public health and the world economy [1]. In *Mycobacterium tuberculosis*, the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains is threatening to make one of the most important infectious diseases untreatable [2]. MDR strains of *M. tuberculosis* are defined as resistant to at least isoniazid and rifampicin, the two most potent first-line tuberculosis drugs. XDR strains of *M. tuberculosis* are MDR strains with additional resistance to fluoroquinolones and to at least one of the three injectable second-line tuberculosis drugs. Recent reports of XDR tuberculosis from KwaZulu-Natal in South Africa have illustrated the dramatic negative impact that drug-resistant tuberculosis can have on patient survival, particularly in areas with high rates of human immunodeficiency virus co-infection [3].

Understanding the ecology of drug-resistant pathogens is essential for devising rational programmes to preserve the effective lifespan of antimicrobial agents and to abrogate epidemics of drug-resistant organisms. Studies in model organisms have shown that drug resistance is often associated with a fitness cost (i.e. reduced growth rate in the absence of the drug) [4]. However, particular drug resistance-conferring mutations can have a variable impact on bacterial fitness, which varies according to the specific resistance-conferring mutation and strain genetic background. Furthermore, compensatory evolution can reduce initial fitness defects caused by particular drug resistance-conferring mutations [5]. In

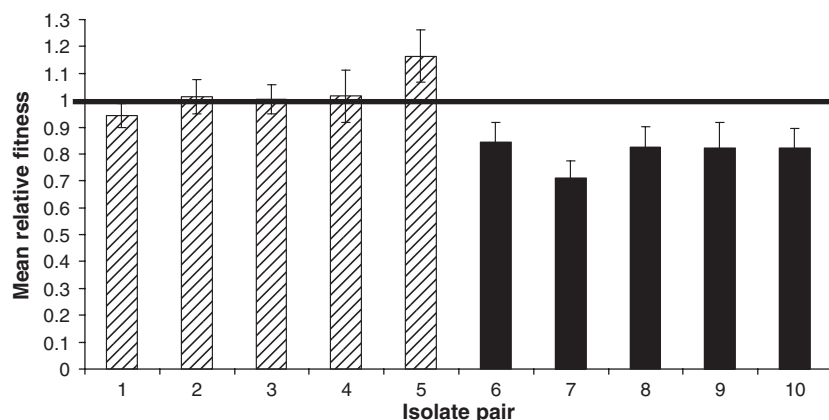
*M. tuberculosis*, mathematical models predict that the future of MDR tuberculosis epidemics will be influenced by strain fitness [6,7]. However, empirical data are lacking. We have used a combination of *in vitro* fitness assays and molecular epidemiological studies to investigate the effect of bacterial genetics on the competitive fitness and relative transmission of drug-resistant *M. tuberculosis*.

To study the influence of different rifampicin resistance-conferring mutations on the fitness of rifampicin-resistant strains of *M. tuberculosis*, we selected for spontaneous *rpoB* mutants *in vitro* using two different pan-susceptible parental strains [8]. We performed competitive fitness experiments *in vitro*, and found that in these laboratory-derived mutants, rifampicin resistance was universally associated with a competitive fitness cost (Fig. 1). However, the magnitude of this defect was determined by the specific resistance mutation and strain genetic background. For example, whereas the *rpoB* S531L mutant had higher competitive ability than other mutants in both strain backgrounds, the *rpoB* H526D mutation had a different fitness effect in the two strain backgrounds. Interestingly, when we compared clinical strains of *M. tuberculosis* that had acquired rifampicin resistance during patient treatment, we found that four out of five clinical strains with the *rpoB* S531L mutations had no fitness cost as compared to the initial rifampicin-susceptible isolate recovered from the same patient (Fig. 2). This suggests that either the *rpoB* S531L change caused no fitness defect in these particular strain backgrounds, or that the low initial fitness costs associated with the *rpoB* S531L mutation were mitigated by compensatory evolution during prolonged patient treatment. Importantly, *rpoB* S531L, the mutation associated with the lowest fitness cost in the laboratory mutants and no fitness cost in some clinical strains, was also the most





**Fig. 1.** Relative competitive fitness of laboratory-derived rifampicin-resistant mutants of *Mycobacterium tuberculosis*. The relative fitness of the rifampicin-susceptible ancestor is defined as 1 (black line). All rifampicin-resistant mutants had a statistically significant fitness cost as compared to the rifampicin-susceptible ancestor strain (error bars indicate 95% CIs). This cost was less in *rpoB* S531L mutants than in other *rpoB* mutants, irrespective of the strain background. Light grey bars, CDC1551 mutants; dark grey bars, T85 mutants.



**Fig. 2.** Relative competitive fitness of clinically derived rifampicin-resistant mutants of *Mycobacterium tuberculosis*. Four of the five mutants with the *rpoB* S531L mutation (hatched bars) had no fitness cost as compared with their rifampicin-susceptible ancestors. All mutants with other *rpoB* mutations (black bars) had significant fitness defects (error bars indicate 95% CIs).

frequent rifampicin resistance mutation among clinical isolates globally. By contrast, the *rpoB* R529Q mutant, which carried the highest fitness cost of all mutants, has never been observed in clinical settings.

In a second study, we investigated the role of different isoniazid resistance-conferring mutations on the transmission of isoniazid-resistant *M. tuberculosis* [9]. Isoniazid is a prodrug that needs to be activated to its bioactive form by the mycobacterial katalase-peroxidase encoded by *katG* [10]. Deletions or missense mutations in *katG* generally lead to isoniazid resistance by disabling drug activation. Because *katG* helps to protect the mycobacterial cell against host-mediated oxidative stress, strains with disrupted *katG* suffer virulence defects in animal models. However, one particular *katG* mutation, S315T, is associated with a high degree of isoniazid resistance but retains enzyme activity and virulence in mice

[11]. Using a population-based molecular epidemiological approach, we measured the relative transmission of isoniazid-resistant strains of *M. tuberculosis* harbouring different *katG* mutations in San Francisco during a 9-year period [9]. We found that only strains with the *katG* S315T mutation or mutations outside of *katG* were associated with successful transmission. In contrast, strains with isoniazid resistance-conferring mutations likely to abrogate *katG* activity did not cause one secondary case during the whole study period. These results were consistent with the experimental evidence reviewed above, and further demonstrate that in *M. tuberculosis*, different drug resistance-conferring mutations can have variable effects on strain fitness and transmission.

*M. tuberculosis* has a phylogeographic population structure, with different strain lineages being associated with different geographical regions [12,13]. When we compared the distri-

**Table 1.** Association between three *Mycobacterium tuberculosis* strain lineages and three classes of isoniazid resistance-conferring mutations

Mutation	Beijing lineage (N = 36) n (%)	Ancestral lineage (N = 45) n (%)	Euro-American lineage (N = 61) n (%)	OR (95% CI) <sup>a</sup>	p
<i>katG</i> mutations other than S315T	15 (41.7)	2 (4.4)	10 (16.4)	5.6 (2.1–15.1)	<0.001
<i>katG</i> S315T	13 (36.1)	15 (33.3)	31 (50.8)	2.0 (0.94–4.1)	0.0517
<i>inhA</i> promoter –15; c-t	5 (13.9)	21 (46.7)	13 (21.3)	3.8 (1.6–9.0)	<0.001
No/other mutation	3 (8.3)	7 (15.6)	7 (11.5)		

<sup>a</sup>For each lineage, the  $\chi^2$  test was used to compare the proportion of the most frequent mutation with its proportion in the two other lineages combined.

bution of strain lineages in the isoniazid-resistant strains from San Francisco, we found statistically significant associations among the three strain lineages that dominate in San Francisco and three groups of isoniazid resistance-conferring mutations (Table 1). The 'Beijing' lineage was associated with *katG* mutations other than S315T, which were likely to abrogate *katG* activity. The *inhA* promoter mutation, also associated with isoniazid resistance, occurred significantly more frequently among the 'ancestral' lineage, and the 'Euro-American' lineage was associated with the *katG* S315T mutation, which maintains *katG* activity. These associations suggest that genetic differences between the strain lineages could influence their propensity to have different isoniazid resistance-conferring mutations. In particular, the association of the 'Beijing' strains with 'costly' *katG* mutations is intriguing, as it suggests that this strain lineage might be less susceptible to host-mediated oxidative stress. Alternatively, 'Beijing' strains might be better adapted to compensate for the loss, or reduced activity, of *katG* in the context of isoniazid resistance. As a consequence of either of these scenarios, 'Beijing' strains with low-cost isoniazid resistance-conferring mutations such as *katG* S315T may be particularly fit. In support of this possibility, 'Beijing' strains have been associated with resistance to isoniazid in several geographical areas [14].

Taken together, the data from these two studies show that there is a strong selection for drug resistance-conferring mutations associated with low fitness cost in tuberculosis patients, and that bacterial genetics can impact on the fitness and transmissibility of drug-resistant *M. tuberculosis*. Our find-

ings have implications for predicting the future of the MDR and XDR tuberculosis epidemic.

## Transparency Declaration

The author declares no conflicts of interest.

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# Drug-resistant human immunodeficiency virus

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## Abstract

The development of antiretroviral therapy has led to a major reduction in human immunodeficiency virus (HIV)-related mortality. There are now six antiretroviral drug classes, with more than 20 unique antiretroviral drugs. However, HIV drug resistance occurs with all antiretroviral agents. Drug resistance can affect the response to antiretroviral therapy and is associated with increased mortality. The emergence of resistance in persons on antiretroviral therapy and the transmission of drug-resistant HIV strains to newly infected persons are now major public health concerns. Resistant variants that make up as little as 1% of the viral population in an HIV-infected person are clinically important, as they can rapidly grow under drug selection pressure and lead to therapy failure. However, current resistance assays used in the clinic reliably detect resistant variants only if they make up at least 20% of the circulating viral population. Recently, antiretroviral drugs have been developed that can inhibit HIV replication at new sites within the viral life cycle. These new drugs may improve clinical outcomes in persons infected with multidrug-resistant HIV. This review addresses the epidemiology and biological mechanisms of HIV drug resistance and the new approaches to detect and combat HIV drug resistance.

**Keywords:** Antiretroviral therapy, HIV drug resistance, HIV genotyping

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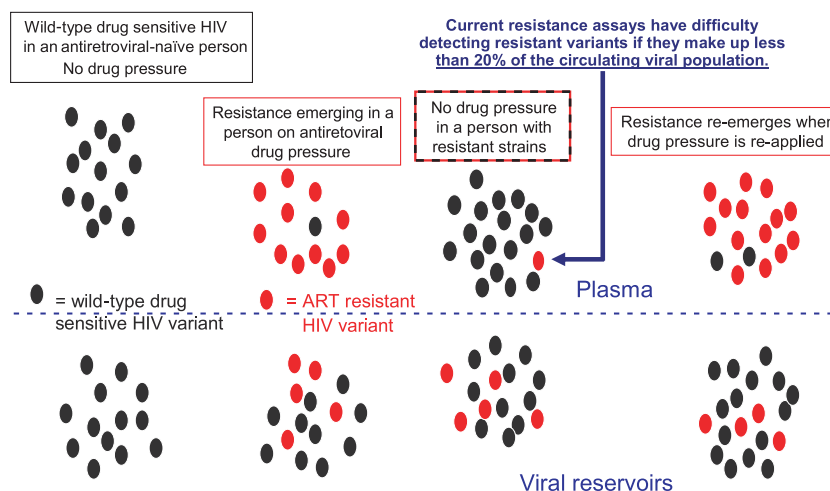
and the new approaches to detect and treat drug-resistant HIV strains.

## HIV Drug Resistance Dynamics

## Introduction

Death rates due to human immunodeficiency virus (HIV) have markedly decreased in regions of the world that have full access to antiretroviral drugs. Currently, there are six classes of antiretroviral drugs that inhibit HIV replication at multiple different sites in the viral life cycle [1]. However, in the face of this advance, there is concern about the development and transmission of drug-resistant HIV strains. HIV drug resistance occurs with every antiretroviral agent. The development of resistance limits the efficacy of all antiretroviral drugs [2,3]. Drug resistance can lead to treatment failure and is associated with increased mortality [4–7]. Therapy for persons infected with multidrug-resistant HIV can be complicated and often leads to difficult patient management issues for clinicians. Furthermore, HIV-infected persons with resistant strains have been shown to continue HIV risk behaviours and to transmit resistant strains to newly infected persons [8]. This article addresses the epidemiology and biological mechanisms of HIV drug resistance

In an untreated HIV-infected person, approximately ten billion viruses are produced each day, and 100 million new cells are infected [9,10]. The poor fidelity of the HIV reverse transcriptase allows for random mutations with each round of HIV replication [9]. HIV has a high mutation rate, such that, theoretically, every possible single-base mutation can occur within the virus each day [9,10]. These evolutionary characteristics make HIV highly responsive to selection pressure from drugs that are not fully suppressive of viral replication. If an antiretroviral regimen does not fully control HIV replication, drug pressure will lead to the selection and preservation of viral variants with increasingly reduced drug susceptibilities [2]. Continued viral replication in the presence of drug pressure allows for the progressive accumulation of mutations that can lead to increased resistance [2,11]. Some antiretrovirals require only a single point mutation to have high-level drug resistance, whereas others require multiple point mutations [3,11]. The number of mutations required to confer resistance contributes to the genetic barrier to resistance [2,3,11]. Resistant variants in the blood can be replaced by wild-type populations when drug pressure is not



**Fig. 1.** Illustration of the emergence and archiving of drug-resistant human immunodeficiency virus (HIV) variants. A drug-resistant viral strain that is the dominant variant when drug pressure is present can become a minority (low-abundance) viral variant in the blood when drug pressure is removed. In this clinical situation, the currently available clinical genotypic resistance assays cannot detect these resistant variants when they fall below a level of c.20% of the circulating viral population. In an HIV-infected person, if an antiretroviral regimen is used which the minority viral variant is resistant to, the variant can multiply under the drug pressure and rapidly re-emerge, causing treatment failure. ART, antiretroviral therapy.

present. This results from a reversion to a wild-type viral genotype from variants with resistance mutations or the outgrowth of wild-type virus from viral reservoir sites (Fig. 1). However, resistant variants can seed reservoir sites (e.g. latent infected T-cells) and re-emerge if that drug is used again (Fig. 1).

### Epidemiology and transmission of drug-resistant HIV strain

The emergence of HIV drug resistance in persons on therapy and the transmission of resistant HIV strains to newly infected persons are now major public health problems. Transmission of drug-resistant HIV to newly infected persons is a function of the type and the frequency of HIV transmission risk behaviours, the penetrance of antiretroviral drugs in a population, the prevalence of drug resistance in those engaging in risk behaviour, and the stability and transmissibility of resistant strains.

HIV-infected persons harbouring drug-resistant strains have been shown to transmit resistant HIV through high-risk HIV transmission behaviours (unprotected sex or the sharing of needles). Our group investigated the prevalence of drug resistance in HIV-infected persons under medical care who continued to engage in high-risk HIV transmission behaviours [8,12]. Unprotected sexual behaviour was reported by 45% of HIV-infected sexually active patients at

some point during an approximately 2-year study period. Of these persons engaging in unprotected sexual events, 31% had HIV drug resistance at the time of a sexual risk event (c.13% with multidrug resistance) [13]. As with other sexually transmitted diseases, there was substantial and complex variation in the distribution of unprotected sexual events and in the detection of resistance over time. These data demonstrated the importance of ongoing risk reduction strategies for individuals undergoing clinical care for HIV [13].

### Burden of drug resistance in HIV populations

In one of the largest HIV drug resistance surveys ever performed in the USA, the estimated prevalence of HIV drug resistance among an adult HIV-positive population that had a detectable HIV viral load was c.75%, with 48% harbouring multidrug-resistant strains [14]. This US survey covered a time period during which many among the infected population had been exposed to the inferior, early antiretroviral regimens commonly used in the mid-1990s. However, many of these HIV-infected persons may have been able to achieve viral suppression with newer antiretroviral regimens. Recent data suggest that the burden of resistance may be declining in resource-rich countries as newer initial antiretroviral therapy regimens achieve better results [15,16]. It is still sobering

that the burden of resistance among HIV populations can be significant, and this underscores the importance of the continued development of new antiretrovirals and new strategies to treat drug-resistant strains [17].

What will be the burden of resistance in resource-poor countries? Often, in resource-poor countries, the identification of a person failing an initial antiretroviral regimen is based on clinical or immunological monitoring [18]. Viral load monitoring, which would allow the early capture of viral escape, is not widely available in these regions, owing to cost and availability. This inability to detect resistance at an early stage, thus prolonging the detection of regimen failure, has raised the concern that more persons may have multiclass-resistant strains, over time, than if such monitoring were available.

Non-nucleoside reverse transcriptase inhibitor (NNRTI)-based regimens are the most commonly used first-line therapies in both resource-rich and in resource-poor countries. The ability to co-formulate three drugs (two nucleoside reverse transcriptase inhibitors (NRTIs) plus an NNRTI) into a single pill and the excellent virological response rates with these combinations have led to their preferred use as first-line therapy in many programmes. Persons failing these first-line antiretroviral regimens can become resistant to a single class or two classes of antiretrovirals. The likelihood of developing multidrug-resistant strains depends on the regimen used and the time for which a person is left on a failing regimen. In the minority of patients who fail an NNRTI-based regimen, resistance to both the NNRTI and nRTI drug classes can occur, resulting in a person harbouring multidrug-resistant strains [7]. It is possible to construct an antiretroviral salvage regimen in these patients when there is broad access to other antiretroviral classes [7]. Unfortunately, this is not an option in all regions of the world. Thus, over time, the burden of resistance in treated populations in many of the developing countries will probably increase.

### HIV drug resistance in the newly infected person

The prevalence of drug-resistant strains in newly HIV-infected persons depends on the survey period and region of the world investigated. The general estimates are that in the parts of the world where antiretrovirals have been widely available for almost 20 years, the rates are approximately 10% (8–15% in the USA and c. 10% in Europe) [17,19,20]. In other parts of the world where antiretroviral drugs have been available for only a limited time, the prevalence is

lower, often <5% [21]. Over time, as antiretroviral 'roll-out' programmes enroll increasing numbers of HIV-infected individuals, the likelihood of the transmission of drug-resistant strains will increase. The WHO recommends that HIV drug resistance surveillance be part of antiretroviral treatment programmes [21]. Surveillance for emergent, and transmitted, drug-resistant strains in these populations will help instruct the programmes and assist in the development of strategies to prevent the development and transmission of drug-resistant strains [21].

The transmission of HIV strains that are resistant to first-line therapy options in developing countries may disproportionately affect these communities. These regions have only limited options for second-line antiretroviral regimens, as many new antiretroviral drug classes that have activity against drug-resistant HIV strains are not readily available, because funds to purchase these newer agents are lacking. Thus, the rates of response to salvage antiretroviral regimens in these regions may not be as robust as those in parts of the world that have access to the full arsenal of antiretroviral drug classes.

### Detection of HIV drug-resistant strains

It is standard practice to monitor HIV drug resistance using either genotypic or phenotypic resistance assays. The use of resistance assays can instruct drug selection, help in patient management, and improve therapy outcomes [1]. However, a major limitation of commercial resistance assays is their inability to detect low-abundance resistant variants that exist as a small portion of the viral population when there is no antiretroviral drug pressure. Standard resistance assays used in the clinic reliably detect resistant variants only if they make up at least 20% of the circulating viral population [1]. Recent data from multiple groups suggest that resistant viral variants that make up as little as 1% of the viral population in a person are clinically important, as they can rapidly grow under drug selection pressure [22,23]. In these studies, low-abundance resistant variants were found in both acutely and chronically infected populations, and the detection of these previously occult resistant variants predicted subsequent treatment failure [22,23]. Thus, new resistance technologies are needed for the clinic to screen for all types of low-abundance resistant variants. Such assays could have greater clinical utility, improve patient care, and might prove valuable in predicting virological responses to new antiretroviral regimens. A major area of investigation in the HIV resistance field is how best to improve the existing assays to achieve this goal.

## Antiretroviral agents to treat drug-resistant strains

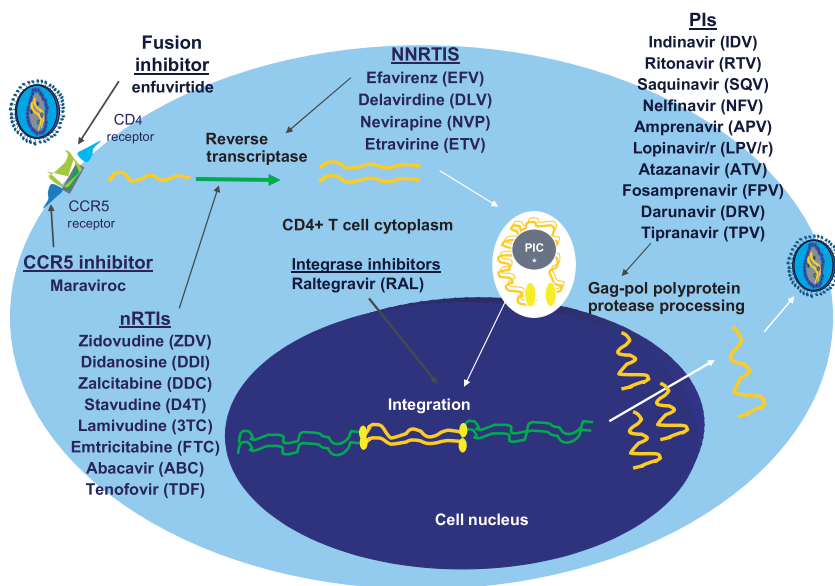
Recently, there have been multiple new antiretroviral agents approved for use in persons infected with drug-resistant HIV. Some of these agents have unique mechanisms of action and inhibit the virus at new sites in the viral life cycle. This advance has allowed clinicians to construct new regimens for persons with multidrug-resistant HIV that have resulted in excellent virological suppression rates [1]. The recently approved antiretroviral agents include new protease inhibitors (darunavir and tipranavir), an NNRTI with activity against drug-resistant HIV strains (etravirine), and antiretroviral classes that inhibit the virus at new sites of the viral life cycle, CCR5 inhibitor (maraviroc) and an integrase inhibitor (raltegravir) (Fig. 2).

The antiretroviral development process has not been synchronous, and the approval of new antiretrovirals was often spread out over many months to years. This staggered release of antiretrovirals led to the use of new agents as functional monotherapy in many patients. New antiretroviral agents were often added to a failing regimen as clinicians were pressed to prevent further immunological deterioration in a person with advanced HIV disease. A single new drug addition to a failing regimen often led to transient virological responses with subsequent therapy failure and the development of even broader multiclass resistance (resistance to the recently added antiretroviral agent in addition to the pre-existing resistance). This clinical practice led to the development of multidrug-resistant HIV strains in many persons.

However, the HIV field has now reached a point in clinical care where, if a person develops multidrug-resistant HIV after initial treatment with an NRTI + NNRTI or NRTI + PI/r (protease inhibitor boosted by ritonavir)-based regimen, a clinician can construct a second-line or third-line antiretroviral regimen that contains two to three new agents with different mechanisms of activity. These new combinations can yield excellent results against multidrug-resistant HIV [1]. Hopefully, this new capability will lead to a lower resistance burden in HIV-infected populations over time (at least in populations that have full access to all antiretroviral drug classes). An important issue for the field will be to bring these newer agents to all who are in need, especially in resource-poor regions, faster than has been done in the past.

## Conclusion

Antiretroviral therapy has dramatically reduced HIV-related mortality. There is an ongoing rapid scale-up of access to antiretroviral therapy in the regions of the globe where HIV-infected populations are in great need. This may lead to the emergence and transmission of drug-resistant strains of HIV, an important problem that we have already witnessed in parts of the world that have had long-standing access to antiretrovirals. The transmission of drug-resistant HIV is an important public health issue in all regions of the world. HIV drug resistance surveillance programmes (such as the WHO HIVDR Program) are needed to ensure the efficacy of antiretroviral therapy programmes and to help direct the choice



**Fig. 2.** Antiretroviral classes/drugs and their site of activity: nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs); non-nucleoside reverse transcriptase inhibitors (NNRTIs); protease inhibitors (PIs); fusion inhibitor; CCR5 inhibitor; integrase inhibitor; \*pre-integration complex (PIC).



of drugs and intervention programmes. Furthermore, new resistance technologies are needed to better monitor low-abundance drug-resistant HIV variants, as these variants can lead to treatment failure. The availability of new antiretroviral drug classes that have activity against drug-resistant strains should improve the clinical outcomes in those infected with multidrug-resistant strains of HIV. Global access to these important new agents will be an important issue for the field.

## Transparency Declaration

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Cover images: On the left, a symbolic image of a bacterial species, sharing a common core (down), that is being diversified to form individual clones (or bacterial ecotypes). The environment in which such a species is located (right part) has a similar structure; the basic environmental features correspond to the core (basic reproductive environment); there is a hierarchy of 'environment ecotypes' corresponding to each bacterial ecotype.