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The Role of Antibiotics and Antibiotic Resistance in Nature

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ABSTRACT

Investigations of antibiotic resistance from an environmental prospective shed new light on a problem that was traditionally confined to a subset of clinically relevant antibiotic-resistant bacterial pathogens. It is clear that the environmental microbiota, even in apparently antibiotic-free environments, possess an enormous number and diversity of antibiotic resistance genes, some of which are very similar to the genes circulating in pathogenic microbiota. It is difficult to explain the role of antibiotics and antibiotic resistance in natural environments from an anthropocentric point of view, which is focused on clinical aspects such as the efficiency of antibiotics in clearing infections and pathogens that are resistant to antibiotic treatment. A broader overview of the role of antibiotics and antibiotic resistance in nature from the evolutionary and ecological prospective suggests that antibiotics have evolved as another way of intra- and inter-domain communication in various ecosystems. This signalling by non-clinical concentrations of antibiotics in the environment results in adaptive phenotypic and genotypic responses of microbiota and other members of the community. Understanding the complex picture of evolution and ecology of antibiotics and antibiotic resistance may help to understand the processes leading to the emergence and dissemination of antibiotic resistance and also help to control it, at least in relation to the newer antibiotics now entering clinical practice.

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INTRODUCTION

Antibiotics are probably one of the most successful forms of chemotherapy in the history of medicine. They have saved many millions of lives and placed the majority of infectious diseases that plagued human history for many centuries under control. Initially, on their introduction into clinical practice in the 1940s, antibiotics were extremely efficient in clearing pathogenic bacteria leading many to believe that infectious diseases would become a problem of the past and would be wiped out from all human populations eventually. However, the emergence and rapid dissemination of antibiotic-resistant pathogens, especially multi-drug-resistant bacteria, during recent decades, exposed our lack of knowledge about the evolutionary and ecological processes taking place in microbial ecosystems. It is now evident that microbial populations possess enormous metabolic diversity, from which they may deploy protective mechanisms allowing them to withstand the selective pressures imposed by their natural environment as well as human interventions such as antibiotics. Revealing the nature and functional role played by antibiotics and antibiotic resistance in various natural ecosystems may help to understand the processes leading to the emergence of antibiotic-resistant pathogens. Finally, armed with knowledge accumulated through many years of genetic, genomic and metagenomic studies and with new concepts about antibiotics and antibiotic resistance, can we now predict the emergence and dissemination of resistance to newly introduced antibiotics?

This review will focus mainly on areas that have contributed to re-evaluation of our conceptual framework about antibiotics and the problem of antibiotic resistance. In particular, the contribution of evolutionary, ecological and functional aspects of antibiotics and antibiotic resistance will be reviewed. In the final section, the practical implications of an attempt to apply these new concepts to the prediction of resistance to a novel antibiotic will be made.

Evolution of antibiotic resistance genes

On an evolutionary scale, the massive explosion of antibiotic-resistant phenotypes in human and animal pathogens is a very recent event that has followed the large-scale production and use of antibiotics in clinical and veterinary medicine, agriculture, aquaculture, horticulture and other human activities. It was thought, initially, that the genetic variability of material in bacterial populations for selection by antibiotics to operate would be through mutations and, as a such, antibiotic resistance would be based largely on target modification and so remain clonal. Indeed, this mechanism is still dominant in the case of resistance to quinolones, rifampin and fosfomycin and it drives the structural evolution of horizontally transferred antibiotic resistance genes such as extended-spectrum beta-lactamases (ESBLs). Mutation-driven antibiotic resistance, however, happens mainly during in-host evolution such as in chronic infections (Maciáal et., 2005) while the purpose of this review is to discuss antibiotic resistance from a broader environmental prospective. The vast majority of antibiotic resistance mechanisms are acquired through horizontal gene transfer from other, often taxonomically very distant, bacteria. Phylogenetic analysis of several groups of antibiotic resistance genes has suggested that genetic material for present-day antibiotic resistance has had a long history of selection and diversification well before the current 'antibiotic era' (Aminov and Mackie, 2007).

Probably the best-documented case of the ancient evolution of antibiotic resistance genes comes from the analysis of β-lactamases. β-Lactams are the most widely used antibiotics in clinical medicine and resistance to β-lactams may become a severe threat because they have low toxicity and are used to treat a broad range of infections (Livermore, 1996). The primary resistance mechanism is enzymatic inactivation through the cleavage of the β-lactam ring by β-lactamases. These enzymes are represented by two unrelated groups, one comprising of three classes of serine β-lactamases, with an active-site serine, and another – of two classes of metallo-β-lactamases, which require a bivalent metal ion to catalyse the hydrolysis (Bush, 1998). Both groups are very ancient and the classes within the groups are diversified to the extent that all traces of homology between the classes at the sequence level are lost (Hall and Barlow, 2004; Garau al et., 2005). Structure-based phylogeny was, however, able to reconstruct the evolution of both β-lactamase groups and establish that these ancient enzymes originated more than two billion years ago, with some serine β-lactamases being present on plasmids for millions of years, well before the modern use of antibiotics (Hall and Barlow, 2004; Garau al et., 2005). Recent work on the evolutionary history of β-lactamase genes in *Klebsiella oxytoca* has suggested that these genes have been evolving for over 100 million years in this host, without concomitant evolution of the antimicrobial resistance phenotype and with the phylogenies of β-lactamase and housekeeping genes being highly congruent in this organism (Fevre al et., 2005). Molecular analysis of β-lactamases in a metagenomic library from 'cold-seep' sediments also showed that most of the diversity of these enzymes is not the result of recent evolution, but is that of ancient evolution (Song al et., 2005). Our own, limited, phylogenetic analysis of class A β-lactamases, with the inclusion of sequences from antibiotic producers such as *Amycolatopsis lactamdurans* and streptomycetes as well as from the environmental bacteria, essentially confirmed these findings (data not shown). Interestingly, the unknown evolutionary forces in apparently antibiotic-free environments may also contribute to the generation of novel diversity in antibiotic resistance genes (Allen al et., 2009). This metagenomic study of Alaskan soil not only uncovered a diverse and ancient collection of β-lactamase genes, but also revealed a novel gene encoding a bifunctional β-lactamase that has never been encountered before.

Recently, we subjected several groups of antibiotic resistance genes conferring resistance to structurally unrelated antibiotics, with different mechanisms of action, to rigorous phylogenetic analyses (Aminov and Mackie, 2007). In general, the history of antibiotic resistance genes can be divided into the macro- and micro evolutionary periods, which can also be defined as the 'pre-antibiotic' and 'antibiotic' periods. The former is characterized by a long history of diversification in natural ecosystems, mostly through duplications and mutations, with a limited contribution of horizontal gene transfer to the processes. What is not known is if these processes have been involved in providing an antibiotic resistance function *per se* years with large-scale production of antibiotics and exertion of a strong selective pressure towards bacteria in various ecosystems. Relatively rare genes that happened to confer antibiotic resistance were once involved in other cellular functions but were selected for the resistance phenotype and mobilized from the environmental genomic reservoirs, with the rapid dissemination into taxonomically divergent commensal and pathogenic bacteria. The process was very rapid on an evolutionary scale and horizontal gene transfer, mediated by mobile genetic elements, played a prominent role in it. or might have served some other metabolic function. The antibiotic era, in fact, was (and still is) a fairly brief evolutionary

experimentation that was conducted during the past 70 Based on the historical evolutionary background, the next question to ask is what possible functional role is played by antibiotics and antibiotic resistance in natural ecosystems?

Ecology of antibiotics and antibiotic resistance

The question of how antibiotic resistance genes are maintained in the environment is contradictory. If the sole functional role of these genes is to confer protection against the lethal concentrations of antibiotics, then selection and dissemination of antibiotic resistance genes should be linked to anthropogenic factors since environmental concentrations of antibiotics in unaffected areas are normally below detection limits and certainly not in the minimum inhibitory concentration (MIC) range for the majority of environmental bacteria. Indeed, this assumption may be supported by cases of detectable antibiotic resistance gene flow from facilities that use antibiotics into the environment (Chee-Sanford al et., 2001; 2009; Koike al et., 2007; Baquero al et., 2008). On the other hand, there are many cases of persistence of antibiotic resistance genes in apparently antibiotic-free environments. In the metagenomic studies of 'cold-seep' sediments and remote Alaskan soil, the presence of diverse β-lactamase genes in both locations has been demonstrated (Song al et., 2005; Allen al et., 2009). Judging by the total amount of environmental DNA sampled and the number of β-lactam-resistant clone encountered, about 5% of average-sized bacterial genomes in this type of undisturbed soil may carry the β-lactamase genes that are readily expressed in *Escherichia coli*. In another example of antibiotic resistance in unaffected environments, the phenotype of more than 60% of the *Enterobacteriaceae* isolates from the pristine freshwater environment was found to be multi-drug-resistant (Lima-Bittencourt al et., 2007). It is now generally recognized that the natural environment harbours a vast diversity of antibiotic resistance genes and some soil bacteria may even subsist on antibiotics using them as their sole source of carbon (D'Costa al et., 2006; 2007; Wright, 2007; Martínez, 2008; Dantas al et., 2008). What, then, is the functional role played by these genes in the environment? The term 'antibiotics' is commonly referred to their therapeutic use and the ultimate goal of any antibiotic therapy is clearing up an infection. Is the same role, in a Darwinian natural selection sense, played by antibiotics and antibiotic resistance in the environment? Indeed, certain strains of fluorescent pseudomonads colonizing euthrophic econiches such as the rhizosphere may suppress soil-born pathogens through the production of diffusible or volatile antibiotics such as phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin, cyclic lipopeptides and hydrogen cyanide (Haas and Défago, 2005). At the same time, several lines of evidence collected in recent years indicate that antibiotic concentrations occurring in natural oligothrophic environments may be too low to exert any lethal effects and, instead, they may play signalling and regulatory roles in microbial communities (Davies al et., 2006; Linares al et., 2006; Yim al et., 2006; Martínez, 2008). If antibiotics are indeed involved in signalling, then what kind of signals they convey? If the signals are related to the environmental conditions, physiological state or other regulatory networks? The most appropriate models to discuss these questions would be the antibiotic producers and regulation of antibiotic synthesis in these bacteria.

Antibiotics as yet another language of communication

The vast majority of commercially available antibiotics are produced by *Streptomyces* spp. (Weber al et., 2003) and the pioneering studies of antibiotic synthesis regulation in the representatives of this genus has resulted in identification of a γ-butyrolactone or A-factor that induces antibiotic production and differentiation in *Streptomyces griseus* (Khokhlov al et., 1967). A pathway for A-factor biosynthesis has been recently proposed (Kato al et., 2007). This group of molecules, represented by three major types, namely 6-keto (6*R*)-hydroxy and (6*S*)-hydroxy types (Nishida al et., 2007), belongs to the quorum-sensing (QS) system, the best-studied prototype of which is the *Vibrio fischeri* QS network (Fuqua al et., 1996). The QS is widespread among bacteria and serves as a language of communication, not only between the bacteria but also in inter-kingdom signalling (Shiner al et., 2005). Similarly to the LuxI/LuxR system of Gram-negative bacteria, the γ-butyrolactone signalling system in *Streptomyces* consists of the γ-butyrolactone synthase, AfsA, and the receptor protein, ArpA (Nishida al et., 2007). During growth, γ-butyrolactones are gradually accumulated in the media and, when they reach critical concentrations, interact with the DNA-binding cytoplasmic receptor proteins, ArpA and its homologues, releasing them and allowing transcription from target genes. The target genes are transcriptional factors that are involved in the production of secondary metabolites such as antibiotics and/or in morphological differentiation (Horinouchi, 2007). The availability of many sequenced bacterial genomes allowed to search for proteins with homology to γ-butyrolactone synthases and receptors. Interestingly, only 10 or 11 probable γ-butyrolactone synthases, all from the representatives of the *Streptomyces* genus, were found while 37–42

putative γ-butyrolactone receptors were found in genomes of bacteria not only from the *Streptomyces* but also from *Kitasatospora*, *Brevibacterium*, *Saccharopolyspora*, *Mycobacterium*, *Rhodococcus*, *Anabaena*, *Nocardia* and *Nostoc* genera (Takano, 2006; Nishida al et., 2007). Thus the behaviour of bacteria in a community may be orchestrated through a small number of γ-butyrolactone producers, with a much larger and diverse audience of signal receivers. Consistent with this, the evolution of γ-butyrolactone synthases and its receptors was not congruent (Nishida al et., 2007). Besides, the ancestral receptors, initially, functioned as regulatory DNA-binding proteins and only later in evolution acquired the γ-butyrolactone-sensing capability (Nishida al et., 2007). As this example demonstrates, the well-known QS signalling network initiates a set of metabolic changes in the community leading to a number of events including differentiation, synthesis of secondary metabolites such as antibiotics and probably other cellular processes.

With few exceptions, such as simple microbiota that occupy extreme ecological niches, functional redundancy is built into many microbial ecosystems to ensure homeostasis and continuous operation even in the case of an acute external stress. The signalling systems in various ecosystems also have a similar level of redundancy, through multiple regulatory networks. The QS system represents one such signalling network and its role in intra- and inter-species communication, as well as in regulation of many aspects of metabolism, virulence, physiology, competence, motility, symbiosis and other functions is described in many excellent reviews. There are a number of other regulatory networks such as two-component systems and various sensors that convey the environmental information to the cell. The languages used in this type of communication, however, have many dialects and are quite specific because they require specific receptors for the signals to be correctly perceived, and only a limited number of microbiota members (roughly fourfold bigger than the original signal producers in the case of antibiotic synthesis; see the ratio of γ-butyrolactone synthases and receptors above) may adequately respond to these signals by reorganizing their cellular processes. Is it that the synthesis of antibiotics is also a signalling mechanism? Then what is the function of this mechanism? Is it in amplification of the initial weak and rapidly decaying signal in the environment to make it stronger and less specific so it is perceived by other members of the community that are not capable of sensing and deciphering the environmental signals themselves? In support of this notion, indeed, the acyl-homoserine lactone quorum signal very rapidly decays in many soil types (Wang and Lead better, 2005). In this scenario, the function of antibiotic resistance may be in attenuating signal intensity similar to quorum quenching in the QS communication (Dong al et., 2001). Indeed, removal of the initial QS signal renders bacteria more sensitive to antibiotics (Ahmed al et., 2007). The negative feedback loop of the secondary signalling system, antibiotics, may then suppress the primary QS signalling network (Tateda al et., 2004; Skindersoe al et., 2008) thus providing the fine-tuning between the two signalling networks.

If antibiotics do indeed play a universal signalling role in natural ecosystems then we would expect to see examples of convergent evolution, for example, in production of the same type of signalling molecules by taxonomically different bacteria employing different biosynthetic pathways. For historical reasons, the search for antibiotic producers was largely confined to streptomycetes (from which the first antibiotics were successfully developed) and, probably because of this, the list of known antibiotic producers is heavily biased towards this group of bacteria. Antibiotic biosynthesis may be a much broader phenomenon in nature and the inclusion of other groups of bacteria and methods of testing in antibiotic screening programmes may uncover the true extent of the environmental antibiome. For instance, β-lactams are a ubiquitous group of antibiotics and are produced by a wide range of bacteria. For the sake of brevity, only the carbapenem group of β-lactams, one of the most therapeutically potent antibiotics currently available (Nicolau, 2008), will be discussed in the context of convergent evolution and signalling and regulation in microbial ecosystems.

The first carbapenem-producing bacterium identified was *Streptomyces cattleya* (Kahan al et., 1972). Structurally similar antibiotics were later described in other *Streptomyces* species as well as in Gram-negative bacteria belonging to the *Serratia* and *Erwinia* genera (Parker al et., 1982). Carbapenem compounds are also produced by a luminescent entomopathogenic bacterium *Photorhabdus luminescens* (Derzelle al et., 2002). Carbapenems are synthesized via a different metabolic pathway from that employed in the classical β-lactam biosynthesis route for penicillins, cephamycins and cephalosporins (Williamson al et., 1985). The genes involved in the synthesis of carbapenems are organized into clusters (McGowan al et., 1997; Cox al et., 1998; Derzelle al et., 2002; Núñez al et., 2003) and although the biosynthetic pathways share some similar enzymes in Gram-positive and Gram-negative producers, they are

substantially different (Coulthurst al et., 2005). These enzymes probably evolved from primary metabolic enzymes in corresponding producers and represent an example of convergent evolution.

The regulation of carbapenem biosynthesis in Gram-negative bacteria also represents an interesting example of its dependence on environmental factors, in particular of QS. Despite the structural similarity between the carbapenems from streptomycetes and a range of Gram-negative bacteria, its regulation is governed by QS signals specific for a given group of bacteria. In *Erwinia carotovora* (recently reclassified as *Pectobacterium carotovorum*) its biosynthesis is regulated by a classical autoinducer, *N*-(3-Oxohexanoyl)-L-homoserine lactone (Bainton al et., 1992). Despite less dependence on antibiotic production from the growth phase and cell density in *Serratia*, antibiotic production is also under a QS control in these bacteria (Thomson al et., 2000). However, a number of other signals are also integrated into the regulatory network in *Serratia* (Slater al et., 2003; Fineran al et., 2005). The complexity of this regulation may reflect the fine-tuning mechanisms regulating the level of antibiotic production in response to the multiple signals perceived by these bacteria from the environment.

Interestingly, cryptic carbapenem antibiotic production genes are widespread in *carotovora E.* and, in laboratory conditions, this phenotype can be suppressed by multiple copies of the apparently mutant transcriptional activator (Holden al et., 1998). It is possible, however, that the antibiotic is synthesized in natural ecosystems but the environmental factors governing its expression are not known. Another possibility might be that if an antibiotic serves as a signalling molecule then the concentrations produced in the environment may be too low to be detected in the MIC and instrumental assays. Much less information is available regarding the regulation of carbapenem biosynthesis in *cattleya S.* but a recent publication suggests that there is an additional, low-level, cross-talk between the thienamycin and cephamycin C pathways in this bacterium (Rodríguez al et., 2008). In another cephamycin C-producing bacterium, *Streptomyces clavuligerus*, antibiotic synthesis is regulated at the primary regulatory level by γ-butyrolactone (Liras al et., 2008). Therefore, the case with carbapenems also supports the hypothesis that cells respond to the initial QS signals and other environmental clues such as *N*-acetylglucosamine (Rigali al et., 2008) and nutrient depletion (Hesketh al et., 2007; Lian al et., 2008), possibly integrating them, by the second level of signalling, through antibiotics. Continuing with the carbapenems as an example, the second-level signalling by the representative of this class of antibiotics, imipenem, at subinhibitory concentrations, involves changes in global gene expression, including β-lactamase and alginate production, in *Pseudomonas aeruginosa* biofilms (Bagge al et., 2004). In Gram-positive bacteria, low-concentration carbapenems are potent inhibitors of L, D-transpeptidases that catalyse the formation of 3→3 peptidoglycan cross-links and bypass the 4→3 cross-links formed by the D,D-transpeptidase activity of penicillin-binding proteins (PBPs) (Mainardi al et., 2007; Lavollay al et., 2008). In *coli E.*, the L, D-transpeptidase homologue is involved in attachment of the Braun lipoprotein to peptidoglycan (Magnet al et., 2007). The lack of the lipoprotein in *coli E.* leads to sensitivity to EDTA, cationic dyes and detergents but no vital cellular functions are affected (Hirota al et., 1977). The question is, how other antibiotic signals are perceived and what kind of phenotypic and genotypic responses they may evoke in other systems?