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THE MULTIPLE ROLES OF ANTIBIOTICS AND ANTIBIOTIC RESISTANCE IN NATURE

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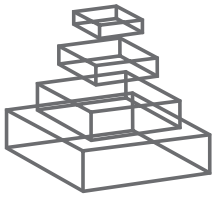

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THE MULTIPLE ROLES OF ANTIBIOTICS AND ANTIBIOTIC RESISTANCE IN NATURE

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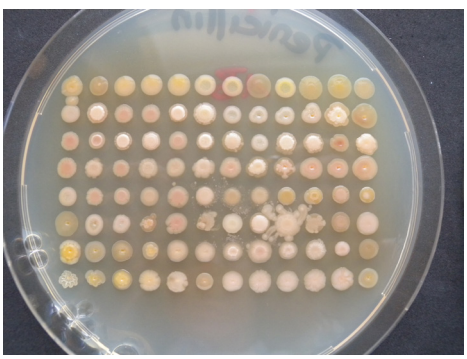

Antibiotic susceptibility testing of bacteria isolated from soil

Antibiotics and antibiotic resistance have most commonly been viewed in the context of human use and effects. However, both have co-existed in nature for millennia. Recently the roles of antibiotics and antibiotic resistance genes have started to be discussed in terms of functions other than bacterial inhibition and protection. This special topic will focus on both the traditional role of antibiotics as warfare mechanisms and their alternative roles and uses within nature such as antibiotics as signals or communication mechanisms, antibiotic selection at low concentrations, the non-specific role of resistance mechanisms in nature: e.g. efflux pumps, evolution of antibiotic resistance and the role of persisters in natural antibiotic resistance.

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The multiple roles of antibiotics and antibiotic resistance in nature

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Keywords: antibiotic resistance, environment, clinical pathogens, soil, plasmids, intrinsic factor

There have been many calls for more information about the natural resistome and these have also highlighted the importance of understanding the environmental resistome in the preservation of antibiotics for the treatment of infections. However, to date there have been few studies which have investigated the roles of antibiotics and resistances outside of the clinical environment. This lack of data also highlights the difficulties faced by microbiologists in designing these experiments to produce meaningful data. Antibiotics and antibiotic resistance have most commonly been viewed in the context of human use and effects. However, both have co-existed in nature for millennia. Recently the roles of antibiotics and antibiotic resistance genes have started to be discussed in terms of functions other than bacterial inhibition and protection. This special topic has focused on both the traditional role of antibiotics as warfare mechanisms and their alternative roles and uses within nature.

The research topic starts with an introduction into antimicrobial resistance in medicine, its linkage to the global environmental microbiota and the many different roles of antibiotics and antibiotic resistance in nature, providing the background to the topic (Cantas et al., 2013). The following chapter discusses the idea that the understanding of antibiotic resistance implies expanding our knowledge on multi-level population biology of bacteria (Baquero et al., 2013). This brings with it inherent problems of designing experimental procedures and standards that can be used in many different microbiomes from human to soil and is discussed further by Walsh (Walsh, 2013). A number of models are proposed to study and understand the biological impact of selection and diversification of antibiotic resistance mechanisms, in particular using the β-lactamases as models (Galán et al., 2013; Patel and Bonomo, 2013; Popowska and Krawczyk-Balska, 2013). The β-lactamases constitute the most widespread mechanism of resistance, at least among pathogenic bacteria, with more than 1000 enzymes identified in the literature. We present some examples of the alternative functions for the multi-drug resistance mechanisms of efflux, that range from bacterial interactions with plant or animal hosts, to the detoxification of metabolic intermediates or the maintenance of cellular homeostasis and also the potential role of extracellular DNA in antibiotic resistance and virulence (Alvarez-Ortega et al., 2013). Bacterial responses to antibiotics may be concentration dependent and so we discuss the different types of interactions mediated by antibiotics and non-antibiotic metabolites as a function of their concentrations and speculate on how these may amplify the overall antibiotic resistance/tolerance and the spread of antibiotic resistance determinants in a context of poly-microbial community (Bernier and Surette, 2013; Lewenza, 2013; Sengupta et al., 2013). The use of antibiotics may also be regarded as pollution. Thus, the widespread use and abuse of antibiotic therapy has evolutionary and ecological consequences, some of which are only just beginning to be examined (Gillings, 2013).

These papers thoroughly review the many different aspects of antibiotic resistance and the roles of antibiotics in nature and link these to the emerging antibiotic resistances of particular importance to the treatment of infectious diseases.

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Investigating antibiotic resistance in non-clinical environments

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There have been many calls for more information about the natural resistome and these have also highlighted the importance of understanding the soil resistome in the preservation of antibiotics for the treatment of infections. However, to date there have been few studies which have investigated the culturable soil resistome, which highlights the difficulties faced by microbiologists in designing these experiments to produce meaningful data. The World Health Organization definition of resistance is the most fitting to non-clinical environmental studies: antimicrobial resistance is resistance of a microorganism to an antimicrobial medicine to which it was previously sensitive. The ideal investigation of non-clinical environments for antibiotic resistance of clinical relevance would be using standardized guidelines and breakpoints. This review outlines different definitions and methodologies used to understand antibiotic resistance and suggests how this can be performed outside of the clinical environment.

Keywords: susceptibility testing, soil, definition of resistance, resistome, guidelines

The World Health Organization World Health Day 2011 highlighted the problems of antibiotic resistance under the title "Antibiotic resistance: no action today, no cure tomorrow". Every year 25,000 people in the European Union die because of a serious resistant bacterial infection, mostly acquired in health care settings[1]. The search for antibiotics, understanding their mechanisms of action and the development and spread of antibiotic resistance has resulted in the development of many industries and novel research areas for over 100 years. However, the number of antibiotics coming to the market over the past 30 years has dramatically declined as many pharmaceutical companies and biotech firms abandoned the search for antibiotics in favor of other pharmaceuticals (Spellberg et al., 2004). Antibiotic resistance has developed over time from resistance to single classes of antibiotics to multi-drug resistance and extreme drug resistance. Until recently, antibiotics and antibiotic resistance were thought of in terms of treatments of infections and the prevention of successful treatment, respectively. The mechanisms of action and resistance have been studied almost exclusively in pathogenic bacteria. It is only in recent years that research in antibiotic resistance has focused on the environment from which the antibiotics were initially extracted: soil microorganisms and the soil ecosystem. With an every decreasing supply of novel antibiotics and increasing resistance the emphasis has turned to defining the natural antibiotic resistome and understanding the ecology and evolution of antibiotic resistance in the non-clinical environment. These recent research focuses are thought to bring answers to help prevent a return to the pre-antibiotic era.

There have been many calls for more information about the natural resistome and these have also highlighted the importance of understanding the soil resistome in the preservation of antibiotics for the treatment of infections (Pruden et al., 2006; American Academy of Microbiology, 2009; Aminov, 2009; Rosenblatt-Farrell, 2009). However, to date there have been few studies which have investigated the culturable soil resistome, in antibiotic producing bacteria *Streptomyces*, and an isolated cave microbiome (D'Costa et al., 2006; Bhullar et al., 2012). Numerous studies have been performed using polymerase chain reaction (PCR) or quantitative PCR (qPCR) to screen the environment for known resistance genes or more recently metagenomics (Volkmann et al., 2004; Chen et al., 2007; Knapp et al., 2008; Lata et al., 2009; Zhang et al., 2011; McGarvey et al., 2012; Popowska et al., 2012). Functional metagenomics have also been used to identify novel resistance mechanisms in soil (Allen et al., 2009; Donato et al., 2010; Torres-Cortés et al., 2011). The identification of antibiotic resistance hotspots and the understanding of the evolution and ecology of antibiotic resistance in the environment are novel areas of research. There have been more reviews written on this topic that research papers to date, which highlights the difficulties faced by microbiologists in designing these experiments to produce meaningful data. We are faced with some fundamental difficulties in assessing and analyzing the environmental antibiotic resistome.

Antibiotic resistance and antibiotic breakpoints have been defined within the context of their medical functions. Clinical breakpoints define bacteria as susceptible, intermediate, or resistant to an antibiotic and are calculated using several factors, including clinical results from studies, wild type minimum inhibitory concentration (MIC) distributions for the relevant bacterial species, antibiotic dosing and pharmacokinetic (PK) and pharmacodynamics (PD) measurements (Clinical and Laboratory Standards Institute [CLSI], 2006; European Committee on Antimicrobial Susceptibility Testing [EUCAST], 2011). This is used as a guide for the clinician to decide how to treat the patient, with antibiotic resistance meaning treatment failure. But what

[1]http://www.who.int/world-health-day/2011/en/

does antibiotic resistance mean out-with the human health or infection treatment context? We need to also define what an antibiotic is in the natural environment, as this too is a term adapted for use in terms of treating bacterial infections.

The soil may be a reservoir of resistance genes, which are already present in human pathogens or which may emerge to increase the current arsenal of antibiotic resistance mechanisms in pathogens. Most antibiotics used in human medicine have been isolated from soil microorganisms. Therefore, soil is thought of as a potential reservoir of antibiotic resistance genes. The presence of antibiotics in soil is believed to have promoted the development of highly specific antibiotic resistance mechanisms in antibiotic producing and non-producing bacteria (D'Costa et al., 2006). This belief is based on studies, which have identified resistance genes such as $bla_{\text{CTX-M}}$, *qnrA*, and bla_{NDM} as originating in the environmental bacteria *Kluyvera* spp., *Shewanella algae*, and *Erythrobacter litoralis*, respectively (Oliver et al., 2001; Nordmann and Poirel, 2005; Zheng et al., 2011). These genes are clinically relevant resistance genes and are currently causing difficulties in the treatment of bacterial infections. The origins of other plasmid mediated resistance genes are still unknown. Anthropocentrism has led to the view that genes such as $bla_{\text{CTX-M}}$, *qnrA*, and bla_{NDM} have evolved in nature as antibiotic resistance genes. However, the true functions of these genes remain to be characterized. By approaching antibiotic resistance in nature not from an anthropocentric viewpoint, but from bacterial evolution and ecological standpoints will we be able to identify the origins and evolution of such genes.

While soil may be a reservoir of antibiotic resistance genes, but not all resistance mechanisms are necessarily a threat to the continued use of antibiotics in all pathogens. Intrinsic resistance is a characteristic of almost all isolates of the bacterial species (Leclercq et al., 2011). Intrinsic resistance occurs when the antimicrobial activity of the drug is clinically insufficient or antimicrobial resistance is innate, rendering it clinically ineffective (Leclercq et al., 2011). The commonly believed theory of the role of the soil resistome is that antibiotic production and resistance co-exist in soil bacteria, as demonstrated by studies of antibiotic biosynthetic pathways and genome analysis (Cases and de Lorenzo, 2005; D'Costa et al., 2007). The theory is that without the resistance gene the antibiotic producing bacteria would self-destruct, on production of the antibiotic. However, Davies and Davies (2010) pointed out that this theory remains to be proven. In order to understand the importance of soil as a potential reservoir of antibiotic resistance mechanisms we need to investigate these theories. The soil may be a reservoir of antibiotic resistance genes, but we need to ask which resistance mechanisms are relevant to clinical antibiotic use?

The human pathogen is not the ancestral home of antibiotic resistance as they have developed to infect humans, not to live in soil with antibiotic producers. Therefore, if soil is the natural reservoir of antibiotic resistance the most important resistance mechanisms are those that can transfer from soil bacteria to pathogenic bacteria. Thus, we need to study soil bacteria as a reservoir of antibiotic resistance with relevance to the clinical use of antibiotics. Another reason to study the soil resistome is to understand the roles of antibiotics and resistance in nature: the natural ecology and evolution of resistance. The idea is that by learning more about how resistance has developed over time we can understand antibiotic resistance evolution and spread within patients and also help to predict the future evolution of resistance to existing and novel antibiotics. Our use of antibiotics has changed the course of antibiotic resistance ecology and evolution. The environment does not exist in a separate world to that of humans. There is a constant flow to and from soil, especially in urban and agricultural environments. Human activities such as using antibiotics in the treatment of human and animal diseases or in agriculture, but also pollution and climate change have altered the soil environment. If the soil is a reservoir of antibiotic resistance mechanisms, we need to identify how our actions and climatic change will also affect the soil resistome. The topic of ecology and evolution of resistance in the environment will be discussed in other papers within this special topic review and will not be addressed in this review.

We frequently refer to bacteria as being resistant to antibiotics, but rarely do we consider what that means. Even the most resistant bacterium can be inhibited or killed by a sufficiently high concentration of antibiotic; patients, however, would not be able to tolerate the high concentration required in some cases (Hawkey, 1998). In order to study the antibiotic resistome, we need to know what antibiotic and antibiotic resistance means in terms of soil bacteria. Antibiotic action and resistance up until a few years ago has been studied almost exclusively in terms of human or animal pathogens. We can separate the study of the soil antibiotic resistome into two different contexts:

1. Clinical relevance: antibiotic resistance of relevance to pathogens
2. Natural relevance: ecology and evolution of antibiotic resistance

These separations ensure that when we study antibiotic resistance in clinical terms that we have a definition of antibiotics and antibiotic resistance, as they are used in medicine. I will focus on the clinical relevance for the remainder of this review. The term antibiotic was described by Waksman (1973) as a description of a use, a laboratory effect or an activity of a chemical compound. Davies and Davies (2010) defined an antibiotic as any class of organic molecule that inhibits or kills microbes by specific interactions with bacterial targets, without any consideration of the source of the particular compound or class. Antibiotic resistance from a clinical viewpoint has been defined by the European Agency for the evaluation of medicinal products, as microbiologically resistant or clinically resistant[2].

Microbiological resistance:

"Resistant microorganisms from a microbiological point of view are those that possess any kind of resistance mechanism or resistance gene." This definition is quantified using MIC data and breakpoints for the antibiotics.

Clinical resistance:

"The classification of a bacteria as susceptible or resistant depends on whether an infection with the bacterium responds to therapy."

[2]http://www.ema.europa.eu/docs/en_GB/document_library/Report/2009/10/WC500005166.pdf

Clinical resistance is a complex concept in which the type of infecting bacterium, its location in the body, the distribution of the antibiotic in the body and its concentration at the site of infection, and the immune status of the patient all interact. The difficulty arises when we try to apply these definitions to soil bacteria or non-pathogenic bacteria, where no breakpoints exist. If we identify a novel resistance mechanism in a soil bacteria, will this resistance gene cause resistance in a human pathogen? The antibiotic resistance definition of most relevance in both clinical and non-clinical environments is that of the WHO:

Antimicrobial resistance is resistance of a microorganism to an antimicrobial medicine to which it was previously sensitive.

In order to define antibiotic resistance in non-clinical environments we need to address the specific context and define what is meant by sensitive. In terms of clinically relevant antibiotic resistances, we can define resistance in all contexts as bacteria containing a known resistance gene or those, which are no longer inhibited at the site of infection. These bacteria would then be considered resistant to the respective antibiotics. Defining an antibiotic sensitive bacteria is a more complex task, especially with respect to bacteria inhabiting different environments. A bacteria defined as sensitive may be so in soil, but in the presence of clinically relevant antibiotic concentrations, may be highly mutable or have inducible resistance mechanisms. Thus, a sensitive bacteria is one, which would not be readily selected in the presence of higher concentrations of antibiotic than those concentrations in the environment. If an environmental species in a particular place increases its MIC to a certain antibiotic along a limited period of time it can be considered that it has become "more" resistant or less susceptible. However, defining resistance by the presence of known resistance genes is limiting the search for resistance to those already characterized. Thus, we propose that such resistance genes be separated into pR-genes, potential resistance genes or pre-resistance genes and aR-genes, genes known to produce an antibiotic resistance phenotype in bacteria capable of survival and integration into the human or animal microbiotia.

How do we decide which resistances are relevant? In clinical practice we know that *Pseudomonas aeruginosa* is intrinsically resistant to ampicillin due to chromosomally mediated AmpC, efflux and impermeability. Therefore, clinical *Pseudomonas aeruginosa* are not tested for susceptibility to ampicillin as it is not used to treat *Pseudomonas aeruginosa* infections. However, if soil is described as a potential reservoir of resistance genes, which can move from the chromosome to mobile elements and then to pathogens we need to test all of the bacteria, not just the pathogens or antibiotic producers. If we identify a penicillin resistant *Pedobacter* species, does this mean that *Pedobacter* contains a potential novel resistance gene or, like *Pseudomonas aeruginosa* it is mediated by intrinsic resistance or that it is only or importance if it can be expressed in pathogenic bacteria?

Novel resistance genes may be identified using functional metagenomics; total soil DNA is extracted, digested, and ligated into a vector. The vector is transferred into a bacterial host, e.g., *Escherichia coli* and the functional characteristics are measured using clinical breakpoints (Handelsman, 2004). However, in hospitals antibiotic resistance is generally tested using MIC or disk diffusion assays. A definition of antibiotic resistance for antibiotic susceptibility testing of bacteria in non-clinical environments is required. The culture-based studies of antibiotic resistance in soil bacteria to date have defined antibiotic resistance as growth at 20 mg/L (D'Costa et al., 2007; Bhullar et al., 2012). This arbitrary definition is based on the use of 20 mg/L as the breakpoint concentration in the initial soil resistome study of *Streptomyces* species (D'Costa et al., 2007). This definition is used for all bacteria and all classes of antibiotics.

The clinical breakpoint of an antibiotic is determined by combining the relevant factors in setting breakpoints for antimicrobial agents and consist of, as defined by the European Committee on Antimicrobial Susceptibility Testing:

1. Available formulations
2. Standard and maximum dosing
3. Clinical indications and target organisms
4. MIC distributions for individual species
5. Pharmacokinetic (PK) data in humans
6. Pharmacodynamic (PD) data
7. Information from modeling processes
8. Clinical data relating outcome to MIC values
9. Information on resistance mechanisms, the clinical significance of the resistance mechanisms, and the MICs for organisms expressing the resistance mechanisms

However, where no PK/PD data for antibiotics with a particular species have been generated the breakpoints should be based on epidemiological cut-off (ECOFF) values for the antibiotics[3]. In the case of clinical infections these are limited to antibiotics used to treat the infection. In a recent evaluation of *Pasteurella multocida* by EUCAST the ECOFF values were determined using approximately 250 isolates for the tests and were estimated by visual inspection or statistically calculated (Turnidge et al., 2006). Ideally greater than 250 isolates would be obtained from multiple centers or countries in order to establish a worldwide ECOFF values. Each country could then survey their own multi-center sites in order to identify edaphic influences on the soil resistome. Although the culturable bacteria represent less than 1% of the total bacterial population, within this 1% remains a large number of bacterial species, for which there are no antibiotic breakpoint values. The possibility of culturing many more organisms than this 1% will be provided by the advances in "culturomics" and combining metagenomics data with concurrent sequencing (Lagier et al., 2012; VanInsberghe et al., 2013). In order to define a bacteria as resistant we need to test the MIC distribution within the population and identify the breakpoint. Therefore, in terms of defining antibiotic breakpoints for bacteria from non-clinical environments the ECOFF would be the most appropriate. In order to create breakpoints for these environments we would need to collect and test at least 250 isolates from different locations, in order to have a representative sample. With breakpoints we can set standard guidelines for susceptibility testing of antibiotics in non-clinical environments. Susceptibility testing is the gold standard of antibiotic resistance testing used throughout the world in hospitals. It is a relatively cheap and easy technique with little need for

[3]http://www.eucast.org/

sophisticated or expensive equipment. Therefore, using susceptibility testing would enable the comparison of non-clinical data with clinical data. However, this is limited to culturable bacteria.

Non-culture-based techniques are required to investigate the entire bacterial community. The bacteria that can be grown in the laboratory are only a small fraction of the total diversity that exists in nature. Approximately only 1% of bacteria on Earth can be readily cultivated *in vitro* (Staley and Konopka, 1985; Amann et al., 2001). Therefore, non-culture-based tools such as PCR and metagenomics are required to capture the non-culturable section of the non-clinical antibiotic resistome. However, one disadvantage of these tools are that they are limited to screening for known resistance genes and mechanisms and identification of only the resistance gene rather than being able to investigate the process involved in resistance, as with culturable bacteria. PCR and quantitative PCR have been frequently used to determine the presence of resistance genes in nature and the effects of agriculture on the emergence and spread of resistance. The most frequently used methods to determine the presence of antibiotic resistance in the environment have been PCR detection, microarray detection or real-time PCR detection of known resistance genes (Chen et al., 2007; Koike et al., 2007; Peak et al., 2007; Walsh and Rogers, 2008; Borjesson et al., 2009; Walsh et al., 2010). These techniques are limited to detecting genes, which are known and have been sequenced. More recently functional metagenomics have identified novel resistance genes and antibiotic biosynthesis genes present in environmental bacteria (Donato et al., 2010; Torres-Cortés et al., 2011). The direct applications of sequencing have to date been mostly focused on the elucidation of species variations within the environment (Kohler et al., 2005; Janssen, 2006; Lauber et al., 2009; Shange et al., 2012).

The application of functional metagenomics on soil DNA have identified novel resistance genes and antibiotic biosynthesis genes present in environmental bacteria (Donato et al., 2010; Torres-Cortés et al., 2011). Functional metagenomics is the genomic study, without culturing, of a population of microorganisms (Handelsman, 2004). This approach has been applied to the study of the antibiotic resistome present in environmental samples (Riesenfeld et al., 2004; Donato et al., 2010; Torres-Cortés et al., 2011). The advantages of metagenomics are that the bacterial community can be analyzed for antibiotic resistance genes without the need to culture these organisms and can be used to detect as yet unknown antibiotic resistance genes. The gene's function is expressed as the antibiotic resistance phenotype in the host bacteria. However, the disadvantage lies with the fact that the host bacteria is frequently *Escherichia coli*, which may not be capable of expressing all of the resistance genes, e.g., *strA*.

Antibiotic resistance genes and plasmids have been identified in pristine and agricultural ecosystems using PCR, functional metagenomics and pyrosequencing tools (Pruden et al., 2006; Allen et al., 2009; Heuer and Smalla, 2012). Both novel resistance genes and bifunctional genes were identified in soil from apple orchards (Donato et al., 2010). The revolution in high-throughput sequencing has brought unique opportunities and challenges to the field of environmental microbiology. Since its introduction in 2005, the number of metagenomic libraries in the database has increased year by year and following the quantum leaps of next generation sequencing techniques (Schmieder and Edwards, 2012). Dependent on the sequencing platform, up to 500 Gb of sequencing data can be generated per single run (Shokralla et al., 2012).

The complete characterization of the soil antibiotic resistome requires both culture and non-culture-based approaches. Using defined guidelines and definitions the scientific community can standardize the methodologies required. This has proved very effective in the surveillance of antibiotic resistance, emerging trends, and novel resistance mechanisms in clinical bacteria. The advantage of identifying the bacteria associated with the resistance mechanism, if it is a novel mechanism, is that the entire genetic cascade or pathway leading to resistance can be studied in depth. However, culture-based techniques alone will only describe a small fraction of the bacteria present in non-clinical environments. A combination of culture-based and non-culture-based standardized techniques will provide a wealth of information on the composition of the non-clinical antibiotic resistome. The WHO definition of resistance is the most fitting to non-clinical environmental studies. The ideal investigation of non-clinical environments for antibiotic resistance of clinical relevance would be using standardized guidelines and breakpoints as outlined in this review.

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A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota

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The discovery and introduction of antimicrobial agents to clinical medicine was one of the greatest medical triumphs of the 20th century that revolutionized the treatment of bacterial infections. However, the gradual emergence of populations of antimicrobial-resistant pathogenic bacteria resulting from use, misuse, and abuse of antimicrobials has today become a major global health concern. Antimicrobial resistance (AMR) genes have been suggested to originate from environmental bacteria, as clinically relevant resistance genes have been detected on the chromosome of environmental bacteria. As only a few new antimicrobials have been developed in the last decade, the further evolution of resistance poses a serious threat to public health. Urgent measures are required not only to minimize the use of antimicrobials for prophylactic and therapeutic purposes but also to look for alternative strategies for the control of bacterial infections. This review examines the global picture of antimicrobial resistance, factors that favor its spread, strategies, and limitations for its control and the need for continuous training of all stake-holders i.e., medical, veterinary, public health, and other relevant professionals as well as human consumers, in the appropriate use of antimicrobial drugs.

Keywords: antimicrobial resistance, human and veterinary medicine, environment, soil, wastewater, resistance genes

BACKGROUND

Arguably one of the greatest examples of serendipity in science was the discovery of natural antimicrobials between Alexander Fleming and Ernest Duchesne. Although Fleming generally holds the reputation of the discovery of penicillin in 1928, a French medical student, Ernest Duchesne (1874–1912), originally discovered the antimicrobial properties of *Penicillium* earlier, in 1896. He observed Arab stable boys that kept their saddles in a dark and damp room to encourage mold to grow on them, which they said helped heal saddle sores. Curious, Duchesne prepared a suspension from the mold and injected it into diseased guinea pigs along with a lethal dose of virulent typhoid bacilli and still all animals remained healthy. His work, however, was ignored because of his young age and unknown status (Pouillard, 2002). In a way, with the success of the natural antibiotic penicillin and the synthetic antimicrobial sulfonamides in the first half of the 20th century, the modern antimicrobial revolution began. Since then, new natural antimicrobial compounds were discovered and many semi-synthetic and synthetic antimicrobial drugs were created to combat bacterial infections. Thus, antimicrobials have been extremely important corner stones of modern medicine since the last half of the previous century. Antimicrobial drugs have saved millions of people from life-threatening bacterial infections and eased patients' suffering. Today, the treatment of bacterial infections is once again becoming increasingly complicated because microorganisms are developing resistance to antimicrobial agents worldwide (Pouillard, 2002; Levy and Marshall, 2004; Alanis, 2005; Pallett and Hand, 2010).

A causal relationship has been demonstrated between the increased use of antimicrobials in both human and veterinary medicine, the greater movement of people, as well as domestic and wild animals, the increased industrialization and the increased prevalence of antimicrobial-resistant bacteria (Holmberg et al., 1987; Cheng et al., 2012). Antimicrobial resistance (AMR) was identified in pathogenic bacteria concurrently with the development of the first commercial antibiotic produced by microorganisms, penicillin (Abraham and Chain, 1940). Although the first concerns about drug-resistant bacteria appeared in hospitals, where most antimicrobials were being used

(Levy, 1998), the importance of AMR was already recognized in 1969 both in humans and veterinary medicine as stated in the Swan Report (Swann, 1969). Further research showed that the origin and spread of AMR is, in fact, a very complex problem. Hence, there cannot be a single solution for minimizing AMR; rather a coordinated multi-disciplinary approach will be required to address this issue (Serrano, 2005; Smith et al., 2009). We must also recognize that wherever antimicrobials are used, AMR will inevitably follow.

The purpose of this review is to highlight the problem of resistance to antimicrobials with its consequences, including how the spread of AMR could be limited. We highlight how the numerous useful applications of antimicrobials led to AMR in different ecological locations (**Figure 1**), aiming to unify the many important aspects of this problem. Finally we advocate the need for teaching and continuous training of all stake-holders (i.e., medical, veterinary, public health, and other relevant professionals) as well as human consumers of antimicrobial drugs, in the appropriate use of antimicrobials.

THE HUMAN MEDICINE AND ANTIMICROBIAL RESISTANCE

EMERGENCE OF ANTIMICROBIAL RESISTANCE AND ITS COST

In human medicine AMR is as old as the clinical usage of antimicrobial compounds. Antimicrobial-resistant pathogens have been observed soon after the introduction of new drugs in hospitals where antimicrobials are intensively used (Levy, 1998).

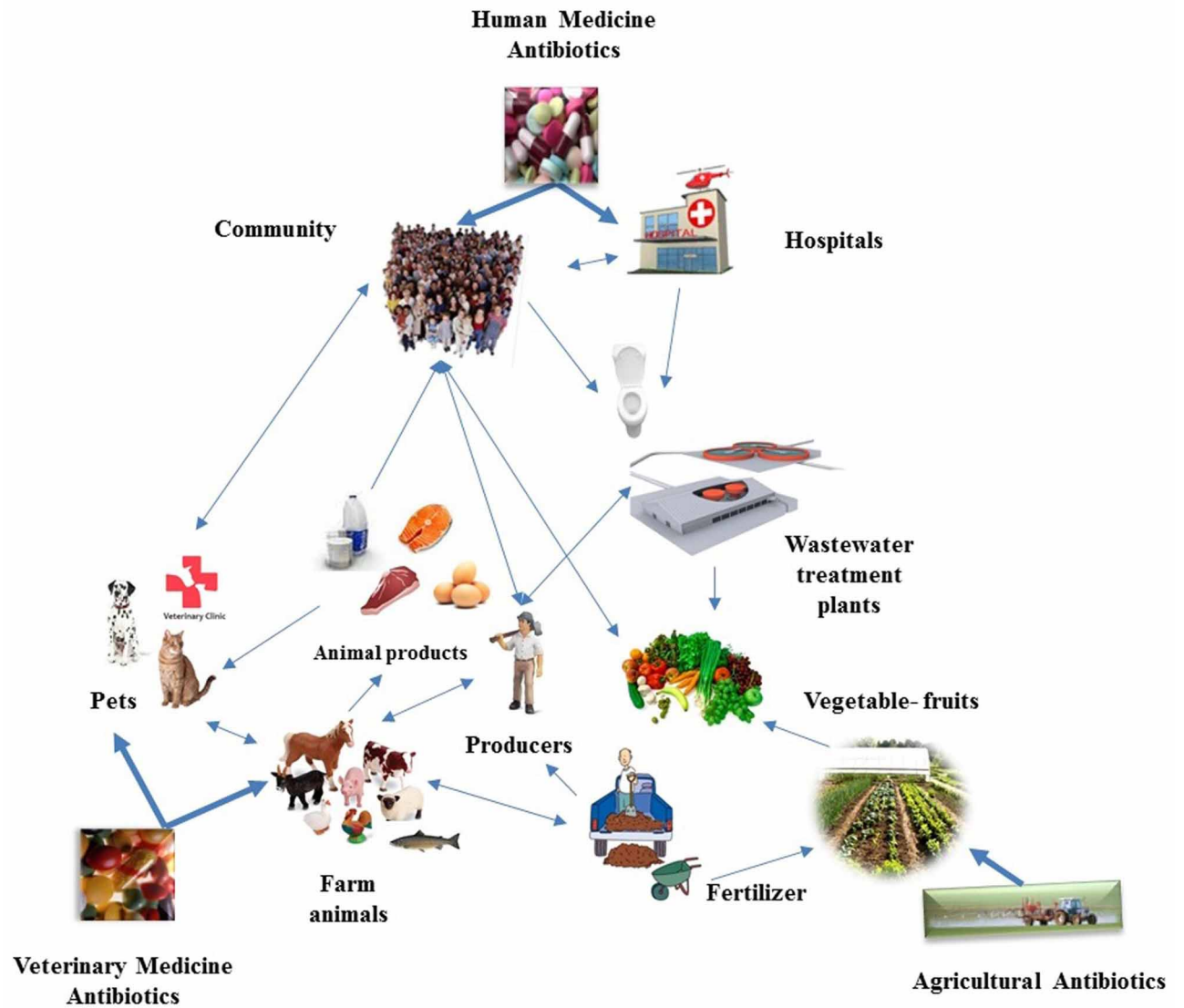

FIGURE 1 | Schematic representation of the complexity of the potential bacterial genetic web of communication between the various microbiotas that are impacted by the use of antibiotics in a wide context. The reservoirs where antimicrobials are applied are also suggested as "hot spots" for horizontal gene transfer. The potentially most important genetic links between the microbiotas of the various reservoirs are showed by arrows. Thick arrows show major selective pressures for selection of antibiotic resistance genes, thin arrows show the significant directions of gene flow. Future research may document unique arrows that must be integrated in the web drawn.

Consequently, AMR in the context of human medicine has dominated the literature for a long time (**Figure 2**). Over the years, and continuing into the present, almost every known bacterial pathogen and numerous human commensals have developed resistance to one or more antimicrobials in clinical use (**Table 1**). Extended-spectrum β-lactamases (ESBL) are the ones most often encountered in the hospital (intensive care) setting. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) have also been found to have a significant nosocomial ecology (Otter and French, 2010). In addition, ESBL positive bacteria and MRSA infections are increasingly detected in the community. Furthermore, the increase in fluoroquinolone resistance due to target-site mutations and the worldwide emergence of plasmid-mediated quinolone resistance genes may represent a major challenge in future given the critical importance of this antimicrobial therapy (Cattoir et al., 2007; Strahilevitz et al., 2009). Carbapenems are the last line of defense against the non-Enterobacteriaceae pathogens, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Brown et al., 1998). However, since the first description of the bla_{OXA} genes, there has been a worldwide increase in the dissemination of new resistance determinants conferring carbapenem resistance. For example, the *Klebsiella pneumoniae* carbapenemase (KPC) type enzymes, Verona integron-encoded metallo-β-lactamase (VIM), Imipenemase Metallo-β-lactamase (IMP) and New Delhi metallo-β-lactamase (NDM), and the OXA-48 type of enzymes have been isolated from a number of bacterial genera irrespective of their geographical distribution (Kumarasamy et al., 2010; Walsh et al., 2011). Carbapenemase resistance mechanisms are found among *Escherichia coli* and *Klebsiella* isolates in hospital settings, and to a lesser extent also in the community, thus healthy human carriers begin to be a concern (Nordmann et al., 2012). Furthermore, carbapenemase-producing organisms have also been isolated from farm animals (Fischer et al., 2012).

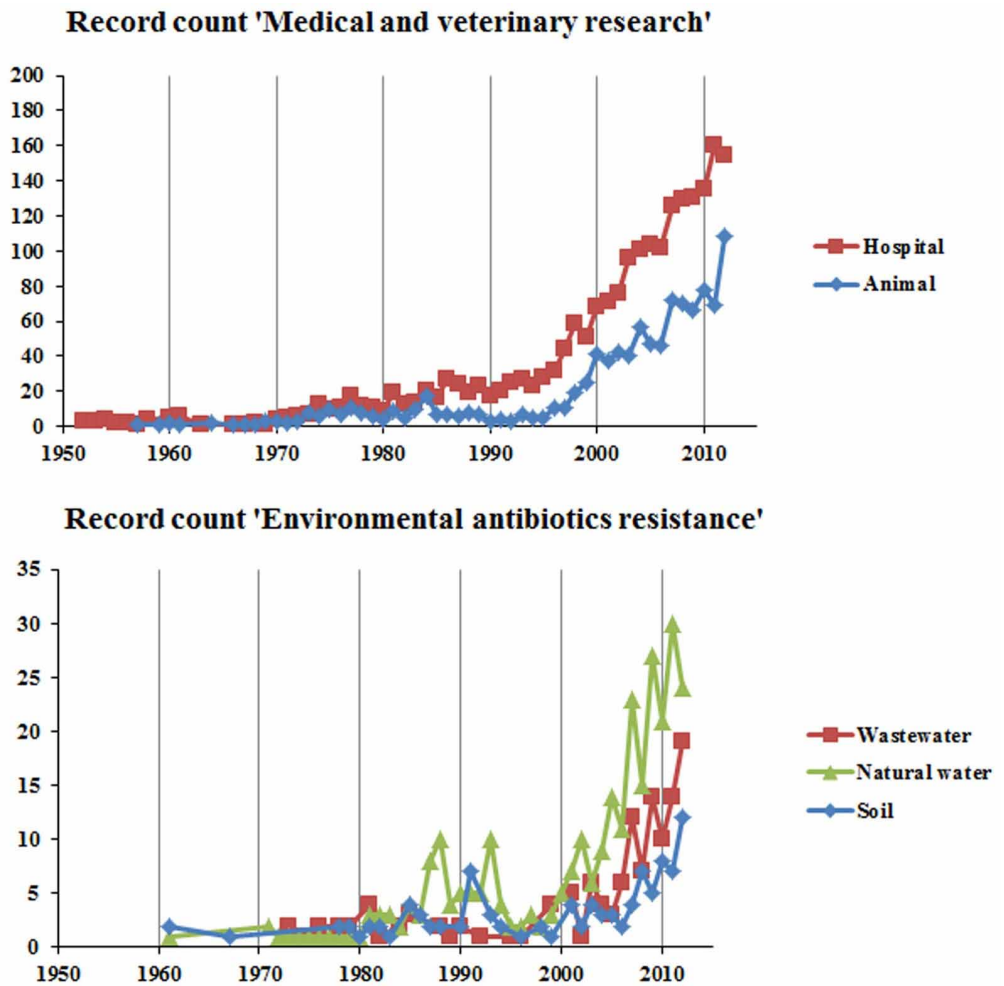

FIGURE 2 | The change in number of antimicrobial resistance related published research papers in different subdisciplines and covering different environments. The data for the graphs were obtained by searching the ISI web of science for publications with titles matching the query terms (antibioti* OR antimicro*) AND resistan* AND the following specific terms: Hospital, (hospital* OR patient* OR clinic*); Animal, (animal* OR veterinary* OR livestock* OR pig* OR cow* OR chicken* OR poultry); Wastewater, (wastewate* OR sewage); Natural water, (wate* OR lake OR river OR ocean OR sea); Soil, (soil* OR sediment* OR rhizosphere*) (Source: http://apps.isiknowledge.com/). The search was performed on 06/03/2013.

Table 1 | Antimicrobial resistance detection in some important pathogens soon after arrival of the "magic bullets" into the market.

Year	Bacteria	Drug resistance	Comments	References
1948	*Staphylococcus aureus*	Penicillin	In British civilian hospitals soon after the introduction of penicillin	Barber and Rozwadowska-Dowzenko, 1948
1948	*Mycobacterium tuberculosis*	Streptomycin	In the community soon after the clinical usage of this antimicrobial	Crofton and Mitchison, 1948
1950's–1960's	*Escherichia coli, Shigella* spp., and *Salmonella enterica*	Multiple drugs		Watanabe, 1963; Olarte, 1983; Levy, 2001
1960's	VRE- *Enterococcus* spp. ESBL- *E. cloacae, K. pneumoniae, E. coli,* MRSA, Q^R- Enterobacteriaceae MDR- *Acinetobacter baumannii, Pseudomonas aeruginosa*	Multiple drugs		Levy and Marshall, 2004; Nordmann et al., 2011

VRE, Vancomycin-Resistant Enterococcus; ESBL, Extended-spectrum β-lactamase; MRSA, methicillin/oxacillin-resistant Staphylococcus aureus; Q^R, Quinolone resistant; MDR, Multi-drug resistant.

HUMAN MOBILITY—THE DIRECT AND INDIRECT IMPACT ON HUMAN PATHOGENS

The increasing cross-border and cross-continental movements of people has a major impact on the spread of multi-resistant bacteria (Linton et al., 1972; Arya and Agarwal, 2011; Cheng et al., 2012). The emergence and global spread of the international clone 1 of penicillin-resistant *Streptococcus pneumoniae* (Klugman, 2002) and the recently occurring New Delhi Metallo-β-lactamase (bla_{NDM}-1) producing *Enterobacteriaceae*, which inactivates all β-lactam antimicrobials, including carbapenems, are good examples. The bla_{NDM}-1 appears to have originated in the Indian subcontinent and subsequently could be found in North America, the United Kingdom, and other European countries by the movement of people (Arya and Agarwal, 2011; Walsh et al., 2011).

The AMR problem remains a growing public health concern because infections caused by resistant bacteria are increasingly difficult and expensive to treat. The consequences of this problem are: longer hospital stay, longer time off work, reduced quality of life, greater likelihood of death due to inadequate or delayed treatment, increases in private insurance coverage and an additional costs for hospitals when hospital-acquired infections occur in addition to the increased overall healthcare expenditure (Roberts et al., 2009; Filice et al., 2010; Korczak and Schöffmann, 2010; Wilke, 2010). Thus, in order to calculate the full economic burden of AMR we have to consider the burden of not having antimicrobial treatment options at all, which in the extreme case would probably cause a breakdown of the entire modern medical system (Alanis, 2005; Falagas and Bliziotis, 2007; Pratt, 2010). In short, everyone will be at risk when antimicrobials become ineffective and the threat is greatest for young children, the elderly, and immune-compromised individuals, such as cancer patients undergoing chemotherapy and organ transplant patients (Tablan et al., 2004).

THE VETERINARY MEDICINE AND AGRICULTURE SECTOR

CONSUMPTION AND REGULATION OF ANTIMICROBIAL USE IN ANIMALS

The antimicrobials, used in veterinary medicine were introduced soon after they became available for the treatment of human diseases from the mid-1940's (Gustafson and Bowen, 1997; McEwen, 2006). Even though some drugs are exclusively designed for veterinary use, most belong to the same antimicrobial classes as those used in human medicine with identical or very similar structures (Swann, 1969; Heuer et al., 2009).

Annually, large quantities of drugs are administered to animals in the agricultural sector worldwide to secure a sufficient amount of food (meat, eggs, and dairy products) to feed a rapidly growing world human population (Vazquez-Moreno et al., 1990; Roura et al., 1992; Rassow and Schaper, 1996). As data collection on antimicrobial use in animals was not harmonized to provide reliable and comparable information, and following a request from the European Commission the European Medicines Agency (EMA), the European Surveillance of Antimicrobial Consumption (ESVAC) programme has been created. The ESVAC programme is responsible for collecting, analyzing, and reporting sales data from European countries and developing an organized approach for the collection and reporting of data on antimicrobial use for animals including annual reporting from EU member states (www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/document_listing/document_listing_000302.jsp). During 2007, in 10 European countries the sale of antimicrobial drugs for therapeutic use as veterinary medicine varied from 18 to 188 mg/kg biomass (Grave et al., 2010).

The administration of antimicrobials to food producing animals can have other purposes than treatment, such as: growth promotion (although now totally banned in Europe and quinolones for the poultry industry are banned in the USA), prophylaxis, and -metaphylaxis (Anthony et al., 2001; Anderson et al., 2003; Casewell et al., 2003; Cabello, 2006). Approximately 70% of all the antimicrobials administered in animal farming are used for non-therapeutic purposes (Roe and Pillai, 2003).

In the European Union (EU) the use of avoparcin was banned in 1997. Furthermore spiramycin, tylosin, and virginiamycin for growth promotion were banned from use in 1998. All other growth promoters in the feed of food-producing animals were banned in the EU—countries from January 1, 2006 (http://europa.eu). Data from Denmark showed that animals could be produced at a large scale without the use of growth promoters,

without adversely affecting the production (Aarestrup et al., 2001; Aarestrup, 2005; Hammerum et al., 2007). In the United States of America, politicians are discussing the introduction of a similar ban on the use of antimicrobials in animal husbandry for growth promotion (http://www.govtrack.us/congress/bills/109/s742). Despite these bans, in some parts of the world, medically important antibiotics are still routinely fed to livestock prophylactically to increase profits and to ward-off potential bacterial infections in the stressed and crowded livestock and aquaculture environments (Cabello, 2006; Smith et al., 2009; Ndi and Barton, 2012). Because stress lowers the function of the immune system in animals, antimicrobials are seen as especially useful in intensive confinements of animals (Halverson, 2000). The non-therapeutic use of antimicrobials involves low-level exposure through feed over long periods—an optimal way in which to enrich resistant bacterial populations (Sharma et al., 2008; Kohanski et al., 2010; Alexander et al., 2011; Gullberg et al., 2011).

Various monitoring programs around the world have started monitoring AMR and a range of research activities and interventions have shown that antimicrobial usage has a large effect upon selection of AMR in animal production. A rapid and parallel decrease in resistant *Enterococcus faecium* from pig and poultry has been reported in Denmark after the ban of growth promoters in livestock (Aarestrup et al., 2001). The Norwegian aquaculture industry has produced over one million tons of farmed fish (http://www.ssb.no/fiskeoppdrett_en/) by using only 649 kg of antimicrobials in 2011 (NORM/NORM-VET, 2011). It is evident from the Danish integrated AMR monitoring and research program (DANMAP) and NORM/NORM-VET Report (NORM/NORM-VET, 2011) that reduction of antimicrobial usage with strict policies may still be the safest way to control the development and spread of AMR in this sector in the future.

ANTIMICROBIAL-RESISTANT BACTERIA IN COMPANION ANIMALS AND ANIMAL HUSBANDRY

The use of antimicrobials in animal husbandry has for many years actively selected for bacteria which possess genes capable of conferring AMR (Bastianello et al., 1995; Sundin et al., 1995). Consequently, this aspect has also seen much attention in the literature (**Figure 2**). Despite large differences in methodology, the results of most relevant scientific studies demonstrate that not long after the introduction of antimicrobials in veterinary practice, resistance in pathogenic bacteria, and/or the fecal flora was observed (Caprioli et al., 2000; Jean-Louis et al., 2000). In particular an increased emergence of pathogenic bacteria resistant to antimicrobials has occurred in members of the genera *Salmonella*, *Campylobacter*, *Listeria*, *Staphylococcus*, *Enterococcus,* and *Escherichia coli.* Some resistant strains of these genera are propagated primarily among animals but can subsequently infect people as zoonotic agents (Levy, 1984; Corpet, 1988; Marshall et al., 1990; Giguêre et al., 2007).

In veterinary medicine the use of antimicrobials in companion animals such as pets and horses is restricted to therapeutic purposes only. Companion animals are increasingly treated as family members, in the context of applying advanced antimicrobial treatments to their infectious diseases. For instance, skin infections caused by staphylococci in dogs with or without underlying allergic reactions result in an increasing use of semi-synthetic penicillins because of the ineffectiveness of penicillin against penicillinase producing *Staphylococcus pseudintermedius* (Yoon et al., 2010). Moreover, emerging methicillin-resistant *Staphylococcus pseudintermedius* (MRSP), methicillin-resistant *Staphylococcus aureus* (MRSA), and ESBL producing *E. coli* displaying multidrug resistance has led to increased concern related to AMR in companion animal practice (Bannoehr et al., 2007; Wieler et al., 2011). Increased antimicrobial resistance development and spread in companion animals due to irrational antimicrobial usage, especially overprescribed broad spectrum antimicrobials without precise diagnostics, inevitably cause (1) animal health problem (increased mortality and morbidity), (2) economical problem to the owner (more visits-therapies and prolonged hospitalization), (3) economical problem to the veterinarian (possible loss of customers and high costs for hospital decontamination), and (4) human health problems (risks of zoonotic transmission). Because of this threat small animal veterinarians should prescribe broad spectrum antimicrobials after culturing and educate pet owners to handle infected-antimicrobial treated animals with precaution.

However, emergences of resistance toward antimicrobials which are critically important for human therapy are the most worrisome. These include the recent emergence of ESBL producing and carbapenemase positive *Enterobacteriaceae* bacteria in animal production (Horton et al., 2011), the emergence of farm associated MRSA ST398 (the main pig associated clone) (Cuny et al., 2010; Kluytmans, 2010; Weese, 2010) and of plasmid-mediated quinolone resistance in animal isolates and food products (Poirel et al., 2005; Nordmann et al., 2011). Unfortunately, there are several examples in the literature that show that these are already widespread in Europe and other parts of the world and have a large impact on human health (Angulo et al., 2004; Heuer et al., 2009; Forsberg et al., 2012).

Aquaculture (fish, shellfish, and shrimp farming industries) has developed rapidly in the last decade and has become an important food source (FAO, 2010). Fish pathogenic bacteria often produce devastating infections in fish farms where dense populations of fish are intensively reared. Although modern fish farming relies increasingly on vaccination and improved management to avoid infections (Markestad and Grave, 1997; Midtlyng et al., 2011), still many bacterial infections in fish are regularly treated with antimicrobials in medicated feed or by bath immersion. The most widely used drugs are fluoroquinolones, florfenicol, oxytetracyclines amoxicillin and sulfonamides (Cabello, 2006; Gräslund et al., 2003; Holmström et al., 2003; Primavera, 2006; Soonthornchaikul and Garelick, 2009). By now, most of the fish pathogenic bacteria from fish farms with a history of infections have developed AMR (Colquhoun et al., 2007; Lie, 2008; Sørum, 2008; Farmed Fish Health Report, 2010; Shah et al., 2012a). Furthermore, in some areas of the world, particularly in South-East Asia, integrated farming is a common practice where organic wastes from poultry and livestock are widely used in manuring the fish farms (Hoa et al., 2011; Shah et al., 2012b). It has been reported that antimicrobial residues present in the poultry and livestock waste has provided sufficient selection pressure for the selection of AMR genes,

increasing the complexity of transmission of bacteria, and resistances between the livestock and aquatic environment (Petersen et al., 2002; Agersø and Petersen, 2007; Hoa et al., 2011; Shah et al., 2012b).

AMR has been detected in different aquatic environments and some resistance determinants have been found to originate from aquatic bacteria. A good example is the recently emerging plasmid-mediated quinolone resistance determinants from the *qnr* family (Ash et al., 2002; Picao et al., 2008) and CTX-M from aquatic *Kluyvera* spp. (Decousser et al., 2001; Rodriguez et al., 2004; Ma et al., 2012). In addition, epidemiological and molecular data indicate that some fish pathogens such as *Aeromonas* are able to transmit and share AMR determinants with bacteria isolated from humans such as *E. coli* (Rhodes et al., 2000; Sørum, 2006). Similarly, the fish pathogen *Yersinia ruckeri* have been reported to share AMR plasmid and AMR genes with the bacterium causing human plague (Welch et al., 2007).

ANTIMICROBIAL USE IN PLANT AGRICULTURE

Streptomycin and oxytetracycline are routinely used for the prophylaxis of fire blight disease (causative agent *Erwinia amylovora*) in apple and pear orchards. Streptomycin use is strictly controlled within the EU and is only authorized for use on a yearly basis. However, streptomycin use in plant agriculture in the USA has been replaced by oxytetracycline, due to streptomycin resistance development among *E. amylovora* in the apple orchards. Oxolinic acid had been reported to be used in Israel against fire blight and against rice blight in Japan (Shtienberg et al., 2001). Gentamicin is used in Mexico and Central America to control Fire Blight and various diseases of vegetable crops (Stockwell and Duffy, 2012). However, the role of antimicrobial use on plants is, knowing the AMR crisis in human medicine, the subject of debate (McManus et al., 2002).

DISSEMINATION OF ANTIMICROBIAL-RESISTANT BACTERIA THROUGH FOOD AND FOOD PRODUCTION

Resistant bacteria can be transferred from animals and plants to humans in many different ways, which can be categorized into three major modes of transmission: (1) through the food chain to people (Roe and Pillai, 2003; Soonthornchaikul and Garelick, 2009), (2) through direct or indirect contact with livestock industry or animal health workers (Levy et al., 1976), (3) through environments which are contaminated with manure in agriculture (http://ec.europa.eu/environment/integration/research/newsalert/pdf/279na4.pdf) and aquaculture (Petersen et al., 2002; Shah et al., 2012b). The environment contains a great variety of bacteria creating an immense pool of AMR genes that are available for transfer to bacteria that cause human disease (Riesenfeld et al., 2004b; D'Costa et al., 2006). The realization of these links sparked the recent interest in the role and dynamics of environmental AMR (**Figure 2**).

In addition, other sources are available. For instance, wild animals may also be carriers of antimicrobial-resistant bacteria (Literak et al., 2011). These animals may have close contact to human or farming areas and/or waste and become colonized with resistant strains (Literak et al., 2011; Nkogwe et al., 2011). Interestingly, animals in remote areas have been found to harbor-resistant bacteria (Zhang et al., 2009; Glad et al., 2010; Lang et al., 2010).

ANTIMICROBIAL RESISTANCE IN THE ENVIRONMENT

MICROBIAL COMMUNITIES IN SOIL AND ANTIMICROBIAL RESISTANCE

Research data shows that in diverse soils from various regions of the world, there is a wide dispersion of AMR. One explanation for this phenomenon is the existence of antimicrobial producing bacteria in soil. The Actinomycetes, which are common soil bacteria (*Streptomyces, Micromonospora, Saccharopolyspora* genus), synthesize over half of all known most clinically relevant antimicrobials e.g., tetracycline, gentamicin, erythromycin, streptomycin, vancomycin, and amphotericin. Bacteria of the genus *Bacillus* also produce antibiotics, e.g., *Bacillus brevis* which producing gramicidin (Baltz, 2007). These antimicrobials now also reach the environment from human and animal therapeutics, through manure, sewage, agriculture, etc. Many retrospective and prospective studies have been performed to assess the emergence and selection of AMR in environmental bacteria. The environment is eventually the largest and most ancient reservoir of potential AMR, constituting the environmental "resistome" (Aminov and Mackie, 2007; Allen et al., 2010; D'Costa et al., 2011). Under such powerful selection pressure, it is not surprising that the soil resistome is so diverse (Knapp et al., 2010). The best example illustrating this is the tetracycline resistome. Tetracyclines are an important class of antimicrobials with desirable broad-spectrum activity against numerous pathogens and the widespread emergence of resistance has had a massive impact on these drugs (Thaker et al., 2010). Opportunistic pathogens ubiquitous in the soil for example, *Pseudomonas aeruginosa, Acinetobacter* spp., *Burkholderia* spp., and *Stenotrophomonas* spp. can combine intrinsic resistance to several antimicrobials with a remarkable capacity to acquire new resistance genes (Popowska et al., 2010). Still, little is known about the diversity, distribution, and origins of resistance genes, particularly among the as yet non-cultivable environmental bacteria. In uncultured soil bacteria, identified resistance mechanisms comprise efflux of tetracycline and inactivation of aminoglycoside antimicrobials by phosphorylation and acetylation (Popowska et al., 2012). In addition, bacteria resistant to macrolides including the new drug telithromycin have been reported from soil (Riesenfeld et al., 2004a). In a study by (D'Costa et al., 2006), 480 strains of *Streptomyces* from soil were screened against 21 antimicrobials. Most strains were found to be multi-drug resistant to seven or eight antimicrobials on average, with two strains being resistant to 15 of the 21 drugs. It was also reported that soil is a reservoir for β-lactamases and these genes, if transferred to pathogens, can then impact human health (Allen et al., 2010). It is supposed that the presence of antibiotics in the environment has promoted the acquisition or independent evolution of highly specific resistance elements. These determinants are located mainly on mobile genetic elements such as plasmids and conjugative transposons, which ensure their spread by horizontal gene transfer. Conjugative, broad-host-range plasmids play a key role in this process (Martinez, 2009; Stokes and Gillings, 2011). Numerous studies have demonstrated that the prevalence of such resistance plasmids in soil is very high

(Götz et al., 1996). Among the plasmids conferring resistance to antimicrobials, representatives of the incompatibility groups P, Q, N, and W have been identified. An example of this type of mobile genetic elements may be the IncP-1 plasmids (Popowska and Krawczyk-Balska, 2013). Results from the scientific literature show that plasmids carrying resistance genes have been identified in pathogenic bacteria of the genus *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, *Aeromonas,* and *Pseudomonas*, the genera that can be found in soil and water (Stokes and Gillings, 2011). These plasmids carry determinants for resistance to at least one heavy metal (Ni, Cd, Co, Cu, Hg, Pb, Zn) and antimicrobials of different groups, i.e., tetracyclines, quinolones, aminoglycosides, sulfonamides, β-lactams, and chemotherapeutics (Sen et al., 2011; Seiler and Berendonk, 2012). Overall these data indicate that soil bacteria constitute a reservoir of resistance determinants that can be mobilized into the microbial community including pathogenic bacteria. Recent studies also indicate a different mechanism of AMR in soil-derived actinomycetes, by engendering mutations in genes encoding the transcriptional and translational apparatus that lead to alterations in global metabolism. This vertically selected AMR includes increased production of secondary metabolites (Derewacz et al., 2013). Very recently evidence for recent exchange of AMR genes between environmental bacteria and clinical pathogens was presented using a high-throughput functional metagenomic approach (Forsberg et al., 2012). In this study it was shown that multidrug-resistant soil bacteria contain resistance gene cassettes against five classes of antimicrobials (β-lactams, aminoglycosides, amphenicols, sulfonamides, and tetracyclines) with high nucleotide identity to genes from diverse human pathogens. Therefore, it is important to study this reservoir, which may contribute to the detection of new clinically relevant AMR-mechanisms and/or the multidrug-resistant pathogens that should be avoided from entering medically important bacteria (Torres-Cortés et al., 2011).

ANTIMICROBIAL RESISTANCE IN AQUATIC ENVIRONMENTS

Water is one of the most important habitats for bacteria, holding complex microbial communities. Not surprisingly, water also contains AMR bacteria. From natural fresh water systems to drinking water, or from sewage to human-engineered water infrastructures, AMR, either intrinsic or acquired, have been reported in aquatic environments worldwide (e.g., Goñi-Urriza et al., 2000; Volkmann et al., 2004; Schwartz et al., 2006; Ferreira da Silva et al., 2007; Böckelmann et al., 2009; Vaz-Moreira et al., 2011; Falcone-Dias et al., 2012). In this respect, given their characteristics, wastewater habitats are particularly important.

WASTEWATER HABITATS AS RESERVOIRS OF ANTIMICROBIAL RESISTANCE

Among the aquatic environments, wastewater habitats represent the most important reservoir of AMR bacteria and genes. This type of water contains human and animal excretions with abundant doses of commensal and pathogenic antimicrobial-resistant bacteria (Yang et al., 2011; Ye and Zhang, 2011, 2013; Novo et al., 2013). Since antimicrobials are not fully degraded in the human and animal body, antimicrobial compounds, their metabolites and transformation products are abundant in urban sewage treatment plants (Segura et al., 2009; Michael et al., 2013). Although proportion of the antimicrobial compounds are transformed and degraded in the environment, the occurrence of these micropollutants is reported worldwide, with antimicrobials of all classes being detected in wastewater habitats in concentrations ranging from ng^{-1} to mgL^{-1} (Michael et al., 2013). Simultaneously, urban sewage and wastewater contain AMR bacteria and other pollutants, such as pharmaceutical and personal hygiene products and heavy metals, whose effects on AMR selection are still not very clear (Graham et al., 2011; Oberlé et al., 2012; Patra et al., 2012; Novo et al., 2013). Often, wastewater treatment is not enough to eliminate the antimicrobial residues entering the system (Michael et al., 2013). The consequence is that such micropollutants, exerting selective pressures, may facilitate the selection of AMR bacteria or the acquisition of resistance genes by horizontal gene transfer (Martinez, 2009). Indeed, the relevance of wastewater habitats to the dissemination of AMR among human pathogens as well as commensal and environmental bacteria is increasingly emphasized (Baquero et al., 2008; Marshall and Levy, 2011; Czekalski et al., 2012; Rizzo et al., 2013). Wastewater treatment plants reduce the load of AMR bacteria, but treated water still carries elevated levels of AMR bacteria, and may select for strains with high levels of multidrug-resistance (Czekalski et al., 2012). Resistance gene abundance in a stream system could be linked to the input of (treated) wastewater and animal husbandry, demonstrating landscape-scale pollution of natural aquatic systems with AMR (Pruden et al., 2012). The currently available literature demonstrates that most of the AMR genetic elements found in clinical isolates are also detected in wastewater habitats, even shortly after they have been reported in hospitals (Szczepanowski et al., 2009; Rizzo et al., 2013). The occurrence of the same AMR genetic elements in different habitats demonstrates the uniqueness of the resistome, mainly due to rapid dissemination processes, demonstrating the urgent needs for an integrated approach.

Ubiquitous bacteria that can live in the environment and are also able to colonize humans are particularly relevant to the spread of AMR in the environment and the implications to human health. Indeed, numerous studies have reported the occurrence of AMR in ubiquitous bacteria isolated from wastewater habitats, which are also recognized as opportunistic pathogens, mainly nosocomial agents. AMR bacteria of clinical relevance which may be found in the environment comprise, among others, members of the genera *Acinetobacter*, *Enterococcus, Escherichia*, *Klebsiella*, *Pseudomonas*, and *Shigella* (Blanch et al., 2003; Reinthaler et al., 2003; Ferreira da Silva et al., 2006, 2007; Watkinson et al., 2007; Novo and Manaia, 2010; Czekalski et al., 2012). In addition, non-cultivable bacteria may also be important either for AMR spread or selection. Indeed, over the last years, the use of culture independent approaches brought additional insights into the abundance and diversity of resistance genes in wastewaters and into the effects of antimicrobials on the bacterial communities (Volkmann et al., 2004; Czekalski et al., 2012; Oberlé et al., 2012; Novo et al., 2013). In particular, several studies presented evidence that in wastewater habitats there is a high potential for horizontal gene transfer, mediated by plasmids and facilitated by integrons (Tennstedt et al., 2003; Szczepanowski

et al., 2008; Moura et al., 2010; Zhang et al., 2011). Despite the importance of wastewater as a reservoir for AMR genes, and the relevance of wastewater treatment to control resistance spread, to date the number of studies that have been published remains relatively low (**Figure 2**).

Nevertheless, over the last decades the knowledge in this area has increased considerably and the importance of wastewater treatment systems for the spread of AMR is unequivocally demonstrated. Therefore, it is now possible to address some specific questions which we expect to be a focus of the research in this area in the coming years. Examples of these issues are (1) the identification of the conditions that may enhance or mitigate the occurrence of horizontal gene transfer and selection of AMR (which pollutants, which concentrations, temperature, pH, hydraulic residence time of wastewater treatment, etc.); (2) the classification and quantification of risk, e.g., the likelihood that an AMR bacterium or gene from wastewater habitats reach humans and causes issues for human health; (3) the improvement of wastewater treatment processes in order to minimize the loads of antimicrobial-resistant bacteria and genes in the final effluent (Dodd, 2012).

Table 2 | Degradation rates of various antimicrobials in soil.

Class of antimicrobial	Degradation [%]	Time [d]	References
Macrolides	0–50	5–30	Thiele-Bruhn, 2003*
Sulfonamides	0–50	22–64	Thiele-Bruhn, 2003
Fluoroquinolones	0–30	56–80	Hektoen et al., 1995; Thiele-Bruhn, 2003
Tetracycline	0–50	10–180	Björklund et al., 1991; Thiele-Bruhn, 2003
Aminoglycosides	0	30	Thiele-Bruhn, 2003
β-lactams	0–50	30	Thiele-Bruhn, 2003
Imidasoles	50	14–75	Thiele-Bruhn, 2003
Polypeptides	12–90	2–173	Thiele-Bruhn, 2003

**This reference does not include the modern macrolides with very long elimination half-lives. For instance, Tulathromycine has an half live (so 50% degraded, not nearly 100%) in soil of 99 days (Pfizer, personal communication 2013).*

THE ANTIMICROBIAL RESISTANCE GENE POOL

AMR genes can be differentiated depending on the genetic event that is required for acquiring an AMR phenotype. These include genes that are acquired by horizontal gene transfer and genes that are present in the bacterial genome and that can encode AMR following gene mutations or activation (Olliver et al., 2005).

AMR features evolve as a consequence of permanent exchange of and ever new recombinations of genes, genetic platforms, and genetic vectors. Many of these genes are not primarily resistance genes, but belong to the hidden resistome, the set of genes able to be converted into AMR genes (Baquero et al., 2009). As evidenced by our discussion above, microbial organisms harboring these genes are present naturally in all kinds of environments, but also released into water and soil from organisms, including humans, where they evolve or increase in abundance under direct selection from exposure to antimicrobials. At the same time, antimicrobials (often at low concentrations), disinfectants, and heavy metals are disseminated into the water as well, and may act as selective factors fostering the evolution of new AMR features (Cantas et al., 2012a,b,c; Cantas et al., unpublished). The rate of degradation of antimicrobials in the environment varies and is dependent on a range of environmental conditions, for example: temperature, available oxygen, pH, presence of alternative sources of organic and inorganic discharges as described in **Table 2**.

HOW TO SLOW DOWN THE SPREAD AND EVOLUTION OF AMR?

In this review we have emphasized that the problem of AMR evolution and dissemination is multifaceted and involves clinical, agricultural, technical, and environmental systems. Similarly strategies to deal with the impending AMR crisis have to take this complexity into account.

The overuse of antimicrobials needs to be limited or reduced in human medicine, veterinary medicine, agriculture, and aquaculture. Ideally, the use of antimicrobials in agriculture should be eliminated. Intensive programs to educate both patients and physicians in reducing antimicrobial overuse should be implemented. Following the analysis more than 500 scientific articles, it has been suggested that the elimination of non-therapeutic use of antimicrobials in food animals, will lower the burden of AMR in the environment, with consequent benefits to human and animal health (FAAIR Scientific, 2002; Swartz, 2002).

Better management techniques and strict legislation in the use of antimicrobials for therapeutic use in humans and in animals will reduce the risk of development of AMR (Cunha, 2002; Defoirdt et al., 2011; Midtlyng et al., 2011). For example, the prevention of nosocomial transmission of multi-drug resistant bacteria is possible with active routine surveillance programs that can identify colonized patients. Numerous studies have demonstrated that such a "search and containment" approach and/or a "search and destroy" approach in which an attempt is made to eliminate carriage of the organism can reduce the incidence of hospital-acquired infections and be cost-saving (Muto et al., 2003).

New management techniques in the animal husbandry, such as organic farming, need to be thoroughly investigated to ensure that these are viable alternatives that help to reduce the potential for selection of AMR bacteria. Samples from organically farmed poultry showed a significantly lower level of AMR in intestinal bacteria such as *E. coli* and *Campylobacter* (Soonthornchaikul et al., 2006). However from organically farmed cattle no significant differences were obtained in microbiological contamination. *E. coli* and *S. aureus* isolates were found to have significantly lower rates of AMR in organically raised cattle (Sato et al., 2005). More studies are needed (1) to determine the reasons of antimicrobial usage in the farms by veterinarians, (2) to compare and update the recommended treatment protocols for veterinarians throughout different countries, (3) to evaluate the impact of other factors other than AMR development in bacteria: e.g., immune response-stress has been indicated to correlate with resistance genetic element shuffling among gut microbiota in different animal models,

such as: atlantic salmon, zebrafish, neonatal piglets, and cats (Cantas et al., 2011, 2012a,b,c, 2013). Animal welfare parameters under intensive production such as stress should be investigated in future studies with regards to control of resistance development in animal husbandry.

Vaccination and improved hygienic measures are among the important cornerstones in controlling infectious diseases and consequently aid in reducing AMR (Potter et al., 2008). The Norwegian aquaculture may serve as a good example by reduction in the use of antimicrobials from around 50 tons in the late 1980's to less than 1000 kg per annum after introduction of effective vaccines against devastating fish diseases like furunculosis and vibriosis (Midtlyng et al., 2011; NORM/NORM-VET, 2011).

The use of pre- and probiotics to improve the health and performance of livestock might be a good alternative to growth promoters. This is an important biological control aiming to reduce outbreaks of infectious diseases and which in turn would minimize the use of antimicrobials in livestock and aquaculture for therapeutic purposes (Verschuere et al., 2000; Callaway et al., 2008).

The issue of dissemination and possible long-term enrichment of AMR and AMR genes in the environment (Knapp et al., 2008) needs to be studied further, with specific regards to the actual risks associated with it. However, taking action is already possible today. For example, several treatment methods for waste and wastewater disinfection and removal of micropollutants, including antimicrobials, are available. These include various chemical disinfections, UV treatment, and membrane filtration. Disinfection and DNA degradation of community based and hospital wastewater may be effective means to reduce AMR release, although more research is required to fully assess the inactivation of resistance genes (i.e., DNA released from lysed cells that may be available for horizontal gene transfer) by these measures (Dodd, 2012). The combined removal of pollutants that are potential selective agents, disinfection, and deactivation of the genetic material, may be a useful strategy to reduce the pollution of environments with resistance factors.

CONCLUSIONS

Inevitably, AMR in medicine has become common place. Bacteria have evolved multiple mechanisms for the efficient evolution and spread of AMR. Meanwhile the new developments of quick and adequate molecular diagnostic techniques for the identification and epidemiological surveillance of genetic determinants of AMR in different hosts and in the environment will enhance the number of control options. We have outlined above a number of potential measures that are enabled by our improved ability to track AMR. However, there seems to be a clear need for action and policy changes. This includes drug licensing, financial incentives, penalties, and ban or restriction on use of certain drugs. Similarly, the prescriber behavior needs to be altered. Animal health and hygiene needs to be improved. In addition, the implementation of microbiological criteria for the detection of certain types of resistant pathogens would be important to control the trade of both food animals and food products. The problem of AMR in human medicine will not be solved if nothing is done to limit the constant influx of resistance genes into the human microbiota via the food chain or contact with the environment. Introduction of antimicrobial compounds into the aquatic environment via medical therapy, agriculture, animal husbandry and companion animals has resulted in selective pressures on resident environmental bacteria. Development of AMR in environmental bacteria has a great impact and may help in explaining how human and animal pathogens acquire resistance features. Besides the role of clinical microbiology laboratories with rapid and accurate detection of a diverse number of pathogens and its drug resistance profiles, robust routine surveillances in an epidemiological frame-work covering the whole livestock "food chain" and the environment need to be taken into consideration. Due to this complexity the control of AMR has to include numerous actions at diverse levels. Future research should focus on finding unknown routes of transfer of AMR between microbiotas of relevance to the food chain and to all microbiotas of importance for bacterial pathogens when they acquire antibiotic resistance genes laterally. Ultimately, even a successful integrative approach on all aspects, can probably only help to slow down the spread of AMR, not prevent it. The development of new generations of antimicrobial should therefore receive equal attention. This is summarized and emphasized in a 12-point action plan against the rising threats of AMR implemented by the European Commission which includes actions in the field of human medicine, animal husbandry, veterinary medicine, authorization requirements for commercialization of human and veterinary drugs and other products, on research, on scientific opinions, and undertaking also actions on the international level in collaboration with the WHO and Codex (http://ec.europa.eu/dgs/health_consumer/does/communication_amr_2011_748_en.pdf, Bush et al., 2011).

The problem of AMR is widespread all over the world, therefore it is not eradicable, but can be managed. Concerted efforts between medical doctors, dentists, veterinarians, scientists, funders, industry, regulators, and multi-disciplinary approaches are needed to track resistance. Furthermore, global monitoring of the antimicrobial drug consumption in human and veterinary medicine and AMR, is an essential part of an overall strategy to inform, educate and get commitment of all parties, including farmers and patients (American Academy of Microbiology, 2009). All these are important measures for the efficient future use of antimicrobials in medicine. All members of society should be conscious of their role and take on responsibility for maintaining the effectiveness of current and future antimicrobials. We believe that future interventions can be successful in minimizing this problem.

AUTHOR CONTRIBUTIONS

L. Cantas defined the review theme, established the interdisciplinary coordination and the collaborations, designed the manuscript, contributed to the data collection, data analysis, and drafting and writing of the manuscript. Syed Q. A. Shah, L. M. Cavaco, C. M. Manaia, F. Walsh, M. Popowska: drafting, writing, and editing the manuscript. H. Garelick and H. Bürgmann: contributed to data analysis, drafting, and writing the manuscript. H. Sørum: contributed to manuscript design, data collection, data analysis, drafting, and writing the manuscript. All authors have contributed to, seen and approved the manuscript.

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Antibiotic resistance shaping multi-level population biology of bacteria

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Antibiotics have natural functions, mostly involving cell-to-cell signaling networks. The anthropogenic production of antibiotics, and its release in the microbiosphere results in a disturbance of these networks, antibiotic resistance tending to preserve its integrity. The cost of such adaptation is the emergence and dissemination of antibiotic resistance genes, and of all genetic and cellular vehicles in which these genes are located. Selection of the combinations of the different evolutionary units (genes, integrons, transposons, plasmids, cells, communities and microbiomes, hosts) is highly asymmetrical. Each unit of selection is a self-interested entity, exploiting the higher hierarchical unit for its own benefit, but in doing so the higher hierarchical unit might acquire critical traits for its spread because of the exploitation of the lower hierarchical unit. This interactive trade-off shapes the population biology of antibiotic resistance, a composed-complex array of the independent "population biologies." Antibiotics modify the abundance and the interactive field of each of these units. Antibiotics increase the number and evolvability of "clinical" antibiotic resistance genes, but probably also many other genes with different primary functions but with a resistance phenotype present in the environmental resistome. Antibiotics influence the abundance, modularity, and spread of integrons, transposons, and plasmids, mostly acting on structures present before the antibiotic era. Antibiotics enrich particular bacterial lineages and clones and contribute to local clonalization processes. Antibiotics amplify particular genetic exchange communities sharing antibiotic resistance genes and platforms within microbiomes. In particular human or animal hosts, the microbiomic composition might facilitate the interactions between evolutionary units involved in antibiotic resistance. The understanding of antibiotic resistance implies expanding our knowledge on multi-level population biology of bacteria.

Keywords: antibiotics, resistance, population biology, multi-level selection, evolution, evolvability, resistome, microbiome

Antibiotics produced by natural organisms play a role in their interactions shaping the lifestyle and homeostasis of bacterial populations and communities (Waksman, 1961; Davies, 2006; Fajardo and Martínez, 2008; Aminov, 2009). Such interactions might be of antagonistic nature as the production of antibiotics serves to inhibit other bacterial populations. Inhibition does not necessarily intend to kill competitive bacterial organisms, but rather prevent undesirable local overgrowth of partners in shared ecosystems. The diffusion of antibiotics in the environment assures an "exclusive zone" at a certain distance from the producer population. At the borders of such a zone, the potentially competing organisms are confronted with very low antibiotic concentrations, probably sufficient to decrease their growth rate, but not to kill the competing neighbor. In this sense, it is highly possible that the production of antagonistic (allelopathic) substances by microorganisms has more a defensive than offensive nature (Chao and Levin, 1981). In addition, mutual inhibition is frequently desirable for the maintenance of healthy species diversity in a particular ecosystem (Czárán et al., 2002; Becker et al., 2012; Cordero et al., 2012). It is of note that natural antibiotic production, decreasing the growth rate of the competing population, not only restricts its local predominance, but also assures that this population is preserved, as antibiotic-promoted cessation of growth is a highly effective system to avoid antibiotic killing.

However, the production of natural antibiotics might only have functions unrelated with inter-bacterial antagonisms. Antagonism might arise in particular contexts as a side-effect of cell-to-cell signaling effects resulting in self-regulation of growth, virulence, sporulation, motility, mutagenesis, SOS stress response, phage induction, transformation, lateral gene transfer, intrachromosomal recombination, or biofilm formation (Goh et al., 2002; Ubeda et al., 2005; Linares et al., 2006; Yim et al., 2007; Martínez, 2008; Couce and Blázquez, 2009; Kohanski et al., 2010; Allen et al., 2011; Baharoglu and Mazel, 2011; Pedró et al., 2011; Looft and Allen, 2012; Looft et al., 2012).

Natural antibiotic resistance modulates the effect of the natural production of antibiotics, so antibiotic production and antibiotic resistance act as two complementary sides of the same process

assuring the homeostasis of microbial populations and communities. In fact, communities with a cohesive habitat association might act as units in terms of antibiotic production and resistance. In these clusters, antibiotics are frequently produced by few bacterial organisms, whereas other members of the club are resistant (Cordero et al., 2012).

As in the case of natural antibiotic production, natural antibiotic resistance might not only focus on "defense against antibiotics" or "self-protection" in antibiotic producers. In fact, this "defense" is frequently a side-effect of other functions of the "natural resistance mechanisms," including nutrition, metabolism, detoxification of noxious substances, and catabolic processes (Dantas et al., 2008; Martínez, 2008; Alvarez-Ortega et al., 2011; Martínez and Rojo, 2011; Qu and Spain, 2011).

The so-called "intrinsic resistome," the ensemble of non-acquired genes and functions normally present in bacterial cells which influence the susceptibility to antibiotics, might account for 3% of the bacterial genome (Fajardo et al., 2008). Obviously, such a huge number of "defensive" genes reflects the unspecific nature of their functions in which antibiotic resistance is concerned (Fajardo et al., 2008; Alvarez-Ortega et al., 2011). That does not mean that these genes were not submitted to horizontal gene transfer before the crisis provoked by the industrial antibiotics pollution, illustrating that besides direct selection by clinical antibiotics other factors contribute to dissemination and maintenance of antibiotic resistance genes in bacterial populations (Biel and Hartl, 1983; Aminov and Mackie, 2007; Mindlin et al., 2008; Allen et al., 2009; D'Costa et al., 2011). In any case, it has been recently suggested that the close identity of resistance genes (and resistance platforms) from clinical strains and environmental strains might indicate recent exchange events (Forsberg et al., 2012). From this perspective, natural antibiotics and antibiotic "resistance" mechanisms have a natural regulatory role in shaping both population biology and evolutionary biology of bacterial organisms. However, the amount of active antibiotic determining this physiological natural "antibiotic environment" is extremely low (Halling-Sørensen et al., 1998). Of course antibiotic-producer organisms are endowed with "mechanisms for self-protection," which have been considered as the origin of modern functions involved in clinical antibiotic resistance (Benveniste and Davies, 1973). However, phylogenetic studies suggest that current clinical resistance genes are not found in antibiotic producers, and its emergence in clinical strains cannot be explained by recent horizontal gene transfer from these organisms. Nevertheless, they might have been historically submitted to duplications and frequent horizontal gene transfer, so that "modern" resistance genes might have evolved along complex evolutionary processes pre-existing the industrial release of antibiotics. In fact many identical "resistance genes" are found in environmental and clinical organisms (Cantón et al., 1999; Forsberg et al., 2012). In any case, what we denominate "antibiotic resistance" for clinical microorganisms is extremely rare in nature; other kinds of "resistance" genes, those of the "intrinsic resistome," able to protect cells from tiny concentrations of natural antibiotics, dominate in the wild environments.

The main problem that we are examining in this manuscript derives from the huge escalation of the amount of antibiotics released into the microbial environments as an effect of anthropogenic action, greatly exceeding the amount of natural antibiotics signaling and controlling the homeostasis of the bacterial world. Moreover, the amount of antimicrobials of anthropogenic origin entering into the environment assures the presence of every possible antibiotic concentration in contact with bacteria. The consequences of such an extensive release of inhibitory and regulatory molecules have an important impact on the population biology and evolutionary biology of bacteria.

POPULATION BIOLOGY OF THE UNITS OF SELECTION

The units of selection define the evolutionary individuals (Lewontin, 1970; Brandon, 1987; Mayr, 1997; Okasha, 2004; Dupré and O'Malley, 2007; Lloyd, 2008; Doolittle and Zhaxybayeva, 2010; Baquero, 2011; Rodriguez-Valera and Ussery, 2012). But what is selected in the case of antibiotic resistance? Possible units of selection in antibiotic resistance are discrete genetic sequences, genes, operons, functional genetic modules, mobile genetic elements (MGEs) as integrons, transposons, integrative–conjugative elements (ICEs), plasmids, or at the cellular and supra-cellular levels, genomes and cells (organisms), clones, clonal complexes, species, communities, and ecosystems. Note that all these possible units belong to different hierarchical levels, ranging from the relatively simple to the complex, as resistance genes are part of integrons, integrons part of transposons, transposons part of plasmids, plasmids part of cells, cells part of clones, clones part of species, and so on. Each unit is a "vessel" for the other(s), affecting not only its potential dissemination but also its rate of introgressive descent and evolution (Doolittle and Zhaxybayeva, 2010; Baquero, 2011; Bapteste et al., 2012; Cordero et al., 2012). The investigation of such a trans-hierarchical landscape clearly requires a multi-level population genetic approach (Baquero and Coque, 2011; Day et al., 2011; Cordero et al., 2012).

What we propose in this work is essentially a mental heuristic exercise. Let us imagine that we are aware of a kind of replicators called genes but we still ignore the existence of cells. We could observe changes in the frequency and variety of genes, and we might consider populations of genes, submitted to evolutionary dynamics and natural selection. If we were only conscious of the existence of transposons, we would establish population biology of transposons. If we considered plasmids, we would refer to the pan-plasmidome, the plasmid population harbored by a particular microbiome or a particular bacterial group (Fondi and Fani, 2010; Mizrahi, 2012). This would apply for every unit of selection. We would of course be able to observe changes in the abundance and variety of each unit involving a resistance trait as a consequence of the presence of antibiotics in the environment. Each unit of selection is a self-interested entity (Rankin et al., 2011b) exploiting the higher hierarchical unit for its own benefit (resistance plasmids exploit successful bacterial clones), but the higher unit might acquire critical traits for its spread because of the exploitation of the lower hierarchy unit (bacterial clones, bacterial communities, or microbiomes might be successful because of resistance plasmids, ICEs, or elements within those; Castillo et al., 2005; Mizrahi, 2012; Novais et al., 2012b). This trade-off of interactions shapes the global population biology of antibiotic resistance. In a certain sense, this multi-level perspective represents a "second line of complexity" in the classic view of antibiotics-driven

natural selection processes, from the selection of a resistant cell to multi-level selections. Such processes should increase the absolute density (number) of all pieces involved in antibiotic resistance, and consequently might favor their interactions and emergence of novel combinatorial patterns (Baquero, 2004). Prediction of which pieces and patterns will evolve is a crucial issue for the management of multiantibiotic resistance, and might be possible if we have the right data (Martínez et al., 2007; Baquero, 2011; Partridge, 2011).

ANTIBIOTICS AND POPULATIONS OF RESISTANCE GENES

Have antibiotics increased the abundance of highly effective resistance genes in the bacterial world? Confronted with antibiotics, bacterial populations might adapt by selecting "more effective" mutants of wild genes endowed with other functions, but providing low level of resistance. Such process is favored by gene duplication, so that wild genes having a "small effect" on resistance could increase in number to increase protection. It is of note that this process might be much more frequent than mutation (Näsvall et al., 2012). A high number of gene-copies might in fact transitorily accumulate during selection, producing a full resistance phenotype. The question is if such gene duplications might contribute to the emergence of novel resistance genes. Once the permanence of a functional copy of a given gene is guaranteed, the second (or *n*-) copy has the evolutionary freedom (liberation from purifying selection) to be modified, eventually leading to a variant or novel gene (Kondrashov et al., 2002; Näsvall et al., 2012). It is of note that not all genes have equal chances of duplication, and certainly there are adaptive genes with a higher potential variability, containing highly variable regions interspersed among well-conserved, "segmentally variable genes," as ABC transporters involved in multidrug resistance (Zheng et al., 2004). Mutational changes in genes, leading to novel resistance genes, are facilitated under circumstances of enhanced mutagenesis in the host strain. Hyper-mutable bacteria ("mutators") are enriched in allelic variants of resistance genes, eventually providing wider resistance spectrum, as in the case of beta-lactamases (Baquero et al., 2005). Indeed organisms with enhanced mutation rates (frequently involving failures in the mismatch repair system) see their possibility of survival increased, and inside these strains, other genes could be modified to provide antibiotic resistance. Note that hyper-mutation and gene variation at large, might result from the effects of the antibiotic themselves (Blázquez et al., 2012).

Indeed, intra- and inter-bacterial gene movement and recombinational events between genetic platforms contribute to the total amount of resistance genes. In fact, the "biological success" of a resistance gene is dependent on its wider genetic context (Walsh, 2006; Wozniak and Waldor, 2010; Bertels and Rainey, 2011). Moreover, hybrid resistance genes resulting from recombinational events are not infrequent in nature (Goffin and Ghuysen, 1998; Maiden, 1998; Novais et al., 2012b). Under antibiotic exposure, bacterial pathogens in humans and animals (and commensals) might fix and further refine acquired resistance genes originated in areas less exposed to antimicrobials, as in the soil (including the rhizosphere!), or water bodies (including sewage!) (Aminov, 2009; Lupo et al., 2012).

If antibiotics have polluted the entire globe, including wild environments, specialized antibiotic resistance genes, identical to those found in hospitals, can be found everywhere else, including the most remote and wild regions (Gilliver et al., 1999; Osterblad et al., 2001; Sjölund et al., 2008; Allen et al., 2009; Quinteira et al., 2011; Stalder et al., 2012). However, the variety of natural bacterial genes that can provide antibiotic resistance in a heterologous host is much larger than that actually found in human pathogens (Dantas et al., 2008). Why only a very small fraction of "resistance genes" present in the "global resistome" have entered in human pathogens is a poorly addressed question. Of course genes from phylogenetically remote organisms should have functional connectivity and concert with the host systems, and that certainly constitutes an important bottleneck for their acceptance (Halary et al., 2010; Martínez, 2011). However, relatively "independent" functionally connected gene clusters (Zheng et al., 2005) determining resistance might be better tolerated and eventually fixed (Popa et al., 2011). In any case, as stated recently (Skippington and Ragan, 2011), the network and evolutionary dynamics that allow the stoichiometric participation of horizontally transferred genes in cellular networks remains poorly addressed, even though new bioinformatic advances have recently been made available (Cohen et al., 2012).

Considering potential sets of "acceptable" resistance genes able to evolve in bacterial populations, eventually only the "fittest genes" resulting from competition among genes might finally reach high densities. Competition is expected to occur mostly among orthologs or paralogs (for instance resulting from recent duplications) occupying the same functional niches (Kondrashov et al., 2002; Francino, 2012). Antibiotics could have enriched the more efficient adaptive genes among competing genes (for instance the more detoxifying ones; Novais et al., 2010b). However, the "fittest genes" are not necessarily those with the best intrinsic activity in terms of providing antibiotic resistance. Different resistance genes impose different biological costs for the host strain. As it was stated above, successful novel resistance genes should be fit in a particular genetic context, that is, the epigenetic compatibility of a new gene with the host genome is critical in the acquisition of resistance (Sánchez and Martínez, 2012). Such fitness bottleneck will select, in combination with the detoxifying efficiency, the novel successful genes.

Alternatively, the quantitative success of a particular gene (as $bla_{\mathrm{TEM-1}}$ in *Escherichia coli*, or *blaZ* in *Staphylococcus aureus*) might result only from a "founder effect" (Livermore, 2000; Martinez et al., 2009), that is, the first gene that by chance conferred a selective advantage in particular conditions was fixed and that resulted in a successful wide spread. This founder effect in human and animal pathogens might have occurred because of multiple selective events in environmental organisms exposed to dynamic landscapes. In fact, founder effects are expected to occur in a continuous and cumulative way (Aguilée et al., 2009). In turn, such emergent events might have been facilitated by changes in environmental conditions (as animal crowding in farms) resulting in local fluctuations in the size of particular bacterial populations, thus favoring acquisition by lateral genetic transfer of adaptive traits from environmental bacteria (Balloux, 2010). Similarly, the changes in the environment, the landscape dynamics, influence the probability of founders fixation, as well as the possibilities

for extinction and re-emergence (Aguilée et al., 2011). It is not impossible that many resistance genes, even those rarely found or never found in the clinical environment, could have also been enriched by environmental antibiotic exposure (Martinez et al., 2009; Sommer et al., 2009).

How can we explain that the same resistance gene of plausible environmental origin (as bla_{CTX-Ms} or bla_{CMYs}) appears to have been captured in separate events by different gene-capturing elements as IS*Ecp*, IS*CR1*, or IS*26*? (Barlow and Hall, 2002; Toleman et al., 2006; Valverde et al., 2009; Partridge, 2011). There is a contemporary enrichment in organisms as Enterobacteriaceae of captured gene(s) with apparently the same function, and a bloom of a diversity of new genes coding for resistance to beta-lactams (Cantón and Coque, 2006; Coque et al., 2008; Poirel et al., 2012). This might be due to a dense interactive field resulting from an in the number of environmental species (donors) where it originated from, as well as an environmental increase in population density of a variety of "good recipients" as *E. coli* or *Klebsiella pneumoniae*. Recipients increase could result from both the augmentation of the total number of Enterobacteriaceae in the gut microbiota of mammals, probably due to antibiotic exposure, and the massive release of human-and animal sewage to the environment (see later).

Other less-successful resistance or pre-resistance genes might not be relevant in the clinical setting, but constitute an increasing reservoir of unpredictable consequences, and undoubtedly might influence the population ecology of bacteria. On the other hand, the selection of variant genes might occur at very low antibiotic concentrations (Henderson-Begg et al., 2006; Gullberg et al., 2011) particularly among natural concentration gradients (Baquero and Negri, 1997; Negri et al., 2000; Hermsen et al., 2012). It can be suggested that the overall increase in the amount of resistance genes on Earth also has a positive effect in maintaining the desirable homeostasis of bacterial populations in a heavily antibiotic-polluted environment.

An interesting point in gene population biology is the question of why a number of resistance genes maintain their full sequence integrity through myriads of replications in spite of an apparently insufficient level of antibiotic selection. Even considering that they are co-selected with genes under active selection (for instance being part of the collection of gene cassettes of an integron), their functionality seems to be better preserved than could be expected. This might suggest that the current function of a number of classic "resistance genes" is something other than antibiotic resistance (exaptation; Alonso and Gready, 2006; Petrova et al., 2011; Sánchez and Martínez, 2012).

Resistance genes tend to be collected in particular clones and clustered in common genetic platforms (Partridge and Hall, 2004; Ktari et al., 2006; Partridge, 2011; Potron et al., 2013), probably following the "genetic capitalism principle," that is, the more resistant clones and the most fit resistance-providing platforms are selected, and then their ability to acquire novel adaptive traits is favored (Lawrence, 1997; Baquero, 2004). As we will see along this review, the extensive antibiotic-promoted selection of resistant bacterial organisms is a selection of the "vehicles" where antibiotic resistance genes are located, and the selection of "vehicles" assures the selection of the genes that they contain. Consequently, the total amount of resistance genes should increase under selection. Interestingly, some "vehicles" (as MGEs are more frequently associated with resistance genes than others. As complementary explanations, we can recall here the founder effect (advantages for the first gene-capturing MGEs), the influence of genes and platforms on the overall fitness of the recipient cells, or the higher prevalence of particular MGEs in the organisms more exposed to antibiotics, heavy metals, biocides, or other ecological stressors (Stokes and Gillings, 2011).

ANTIBIOTICS AND POPULATIONS OF RESISTANCE INTEGRONS

Have antibiotics increased the abundance, in the microbial world, of integrons able to capture resistance genes? Integrons possess a site-specific recombination system able to integrate, rearrange, and express adaptive genes, including antibiotic resistance genes (Mazel, 2006; Partridge, 2011; Stokes and Gillings, 2011; Moura et al., 2012). These genetic platforms are ancient structures (several hundred million years old) that were already involved in the initial outbreaks of antibiotic resistance in the 1950s (Liebert et al., 1999; Mazel, 2006; Revilla et al., 2008). The same type of integrons, now carrying a diversity of antibiotic resistance genes, are preserved in the current bacterial world, and have installed themselves in natural environments along extended periods of time (Petrova et al., 2011; Stokes and Gillings, 2011; Stalder et al., 2012). Integrons evolution often results in the local array of resistance genes, and other genes of adaptive nature (Labbate et al., 2009; Moura et al., 2012; Stalder et al., 2012), which increases the possibilities of their selective multiplication.

In other words, exposure to antibiotics, biocides, or heavy metals and a high multiplicity of other different environmental factors results in an increase of cells containing integrons (Gaze et al., 2005, 2011; Wright et al., 2008; Stalder et al., 2012). Moreover, exposure to different antibiotics (aminoglycosides, beta-lactams, fluoroquinolones, trimethoprim, metronidazole) facilitates extensive gene cassette recombination; occasionally involving the SOS-triggered IntI1 integrase over-expression (Guerin et al., 2009; Hocquet et al., 2012). Other recombinational events (often mediated by ISs) influence shuffling of resistance genes contained in different integrons, giving rise to multi-resistance regions (Partridge, 2011) and further facilitating the evolution and selection of the upper-level host vehicles (Domingues et al., 2012). In fact, integrons are not mobile, but are frequently associated with transposons and/or plasmids and therefore should increase in abundance as a result of conjugation or transposition events mediated by Tn*21*-like and IS*26*-like elements. For instance class 1 integrons located in Tn*402*, which are often part of Tn*501*-like transposons on conjugative plasmids, have greatly contributed to the spread of integrons among γ and β-proteobacteria (Tato et al., 2010; Partridge, 2011).

The frequent association of integrons with a variety of specialized DNA recombination systems enhances both transferability and genetic diversification. For instance, insertion sequences (IS) of the IS*110*/IS*492* family as IS*4321* and IS*5075* (members of IS*1111* subgroup) target the terminal inverted repeats of Tn*21* family transposons (Partridge and Hall, 2003; Novais et al., 2010a). The outcome is the initiation of a non-standard transposition

resulting in only a single copy appearing in the transposed product (Cain et al., 2010; Martinez et al., 2012). Such mobilization of integrons by specialized transposition is a powerful mechanism for integrons spread in both environmental competent and non-competent bacteria (Domingues et al., 2012; Stokes et al., 2012). Also, IS-mediated mobilization of relevant antibiotic resistance genes contained in the integron contributes to enhance gene expression and mosaic genetic diversity, which should be reflected in higher possibilities of dissemination. That is the case for insertion sequences targeting the pseudo-palindromes of integron *attC* sites, as the IS*1111*-*attC* group elements of the IS*110*/IS*492* family (Tetu and Holmes, 2008), or IS*4*-like elements as IS*1999* (Aubert et al., 2006; Post and Hall, 2009; Poirel et al., 2010). Note that the antibiotic-mediated selection of strains, plasmids, or transposons containing integrons necessarily implies selection of IS sequences, and therefore the capture of resistance genes and the combinatorial evolution of resistance platforms. In fact, ISs mobilizing adjacent sequences by rolling-circle (RC) transposition as, IS*Ecp1* and the insertion sequence common regions (IS*CR*s), favor the capture and mobilization of a full series of antibiotic resistance genes leading to complex multi-resistance class 1 integrons (del Pilar Garcillán-Barcia et al., 2001; Garcillán-Barcia and de la Cruz, 2002; Aubert et al., 2006; Toleman et al., 2006; Poirel et al., 2010).

ANTIBIOTICS AND POPULATIONS OF RESISTANCE TRANSPOSABLE ELEMENTS

Have antibiotics increased the abundance of resistance transposable elements in the microbiosphere? Transposable elements encode an enzyme, transposase, which is required for excising and inserting the mobile element. Transposases (revised in Hickman et al., 2010 and references herein) seem to be the most abundant genes in known sequenced genomes and environmental metagenomes (Aziz et al., 2010).

Among transposases, class II dsDNA transposases constitute the most common group, followed by serine and tyrosine recombinases and RC transposases which are linked to different MGEs (IS, composite transposons, class II transposons, bacteriophages, and genetic islands). The wide spread and maintenance of different classes of transposable elements in bacterial populations has been obviously favored by antibiotic selection because of their association with antibiotic resistance genes. However, most of the contemporary antibiotic resistance transposable elements belong to a few families that have been detected in ancient isolates, often linked to alternative functional roles, thus suggesting antibiotic resistance might have overshadowed previous selection forces.

For instance, some transposable elements, as IS or composite transposons, as Tn*5* or Tn*10*, confer growth rate advantages under different conditions of nutrient availability, enabling populations to rapidly adapt to different physiological conditions (Biel and Hartl, 1983; Hartl et al., 1983; Blot et al., 1994). Also, the highly specialized targeting system of Tn*7* able to selectively direct transposition into both mobile and stationary DNA pools (see later), avoids the occurrence of deleterious insertions and allows the host population or community to recruit genes through a variety of mobile DNAs, thus favoring the adaptation of diverse groups of bacteria to survive or adapt to different conditions (Parks and Peters, 2009; Parks et al., 2009). Selection by different ecological conditions and stressors (including antibiotics) multiplies the chances for expansion, recombination, and diversification (Partridge and Iredell, 2012; Seputiene et al., 2012).

The main group within dsDNA transposases corresponds to DDE transposases (designation given due to the presence of a highly conserved catalytic triad of two aspartate (D) and one glutamate (E) residue), originally identified in the retrovirus integrase and having a role during the transfer of the DNA strand. Most IS families use this catalytic reaction for transposition with the exception of IS*1*/IS*110* and the RCR IS*91*-like elements. IS-DDE transposases have been detected in more than 70 bacterial genera, more than one third being iso-elements (>95% of identity at protein level, >90% at DNA level). They are frequently located on plasmids associated with composite transposons containing antibiotic resistance genes (Merlin et al., 2000).

Tn*7* poses an unique fine-tuned regulated transposition array (TnsABCDE) involved in the regulation of transposition (the core machinery coding for TnsA, TnsB, TnsC) and the mobilization of the element (TnsE, TnsD; Parks and Peters, 2009, Parks et al., 2009). Such regulation allows Tn*7* to use two target-site selection pathways and move to different hosts. Tn*7* belongs to a family of MGE that encodes a transposase and an ATP-utilizing protein (TnsC) that controls the activity of such transposase and often its target site selection. Members of the ATP-subunit superfamily comprise widespread AbR transposons that differ in the transposase (also a DDE enzyme) and the number of proteins in the transposition module (for revision see Craig, 2002). Some examples are Tn*1825*/Tn*1826,* Tn*552*-IS*21*, and Tn*402*–Tn*5053* (Craig, 2002). All are widely distributed and they are related with trimethoprim and heavy metals resistance (Kholodii et al., 2003; Partridge, 2011). Please note that in this case trimethoprim resistance is the currently recognizable phenotype, but the genes might have been selected before trimethoprim exposure for other reasons (Alonso and Gready, 2006).

Members of the Tn*3* family are mainly derivatives of transposon subfamilies Tn*3* (Tn*3*, Tn*5393*, Tn*5403*) or Tn*501* (Tn*21*, Tn*501*, Tn*1721*) and all of them were already detectable in ancient bacteria from permafrost (Tolmasky, 1990; Graidy, 2002; Kholodii et al., 2003; Mindlin et al., 2008). Members of the Tn*501* subfamily of Tn*3* transposons could have been enriched by mercury exposure, as they carry mercury-detoxifying genes. These genes probably originated in hydrothermal environments, where geochemically derived mercury is at high concentrations (Boyd and Barkay, 2012). Mercury-transposons provide target sites for Tn*501*-type transposons, and include a diversity of Tn*21*, Tn*1696*, and Tn*501* related elements carrying class 1 integrons. Enrichment of Tn*3*-type transposons by environmental pollutants, as heavy metals, might have contributed to increase the connectivity with organisms and genetic platforms harboring resistance genes, eventually included in integrons (most frequently of class 1), and have converted this family in the "flagship" of floating resistance genes (Liebert et al., 1999; Partridge and Hall, 2004; Partridge and Iredell, 2012).

Beside classic antibiotic resistance gene cassettes, emerging beta-lactamase genes as bla_{VIM}, bla_{IMP} bla_{VEB}, or bla_{GES} are located in integrons on different Tn*501* derivatives (Partridge, 2011). Other widespread "new" beta-lactamase genes have been

directly recruited by host-specific Tn*3*-like transposons as Tn*1440* carrying bla_{KPC} (a Tn*5403*-like transposon from *Klebsiella* which belongs to the Tn*3* subfamily; (Naas et al., 2008).

Such process of gene capture contributes to further increase the number of these transposons, which in turn will facilitate its diversification and eventually novel resistance gene recruitments. Indeed the capture of bla_{TEM1a} by Tn*2* led to, in a landscape of high consumption of aminopenicillins in the 60s and 70s of the past century, a huge amplification of this transposon, now widespread in all environments. Other transposons were less-successful, as Tn*1* (with bla_{TEM-2}), and Tn*3* (carrying bla_{TEM-1a}; Novais et al., 2010a; Bailey et al., 2011), probably because of the reduced numbers and connectivities (influencing success) of the vehicles in which they were located (plasmids, clones; Cain et al., 2010; Novais et al., 2010a; Partridge, 2011).

Transposable elements also increase in number by inserting extra copies in the host genomes. Of course this might cause an "intergenomic conflict," as such insertions might affect chromosomal balance and produce mutations, being so, transposons might be a "bitter–sweet" pill for host bacteria (Toleman and Walsh, 2011). This is why genomes have evolved suppressors limiting transposon spread (Pomiankowski, 1999). Eventually, equilibrium is reached by diminishing the transposition frequency. Successful transposons as Tn*3* have this kind of "transposition immunity" to ensure a maximum of two copies per replicon.

On the other hand, transposition might compete with the host genome replication. Some transposases as that of Tn*7* (and possibly Tn*917*) can bind to "processivity factors" involved DNA replication; competition for this interaction could limit their proliferation. However, a benefit for the transposons appear to be derived from the fact that the interaction of transposases with processivity factors favors "target site selection," so that activation of transposition with Tn*7* (transposon excision and insertion) does not occur until an appropriate target has been identified, most frequently mobile plasmids, providing Tn*7* with a means of spreading to a new host (Parks et al., 2009).

As in the case of integrons, transposable elements are very ancient on Earth, but the very same molecular structures are found in modern resistance-bearing transposons (Bisercić and Ochman, 1993; Mindlin et al., 2005; Vishnivetskaya and Kathariou, 2005; Petrova et al., 2009; Aziz et al., 2010). The acquisition of resistance genes seems to have occurred preferentially by particular transposable elements that were afterward amplified by antibiotics. Interestingly, a number of transposons carrying resistance genes have been recovered from ancient permafrost and seem to have been selected before the antibiotic era (Mindlin et al., 2008). Widespread transposons in our days, as those belonging to Tn*7* or Tn*3* superfamilies, certainly have a very ancient origin. Most probably they were selected by pre-antibiotic forces, increasing their absolute amount, followed by their spread and diversification in different plasmids and organisms. Exposure to early chemotherapeutic agents has reinforced these evolutionary events.

ANTIBIOTICS AND POPULATIONS OF RESISTANCE MOBILE GENETIC ELEMENTS (PLASMIDS, ICEs)

Have antibiotics increased the abundance of plasmids and ICEs carrying resistance genes in bacterial populations and communities? Conjugative plasmids and ICEs are quite similar genomic objects, in fact they appear to be short-term variants of identical backbone elements (Guglielmini et al., 2011); the main difference is that the replication of ICEs occurs only by integration in the host's chromosome (Wozniak and Waldor, 2010). For instance a close relationship resulting from a common phylogeny can be found between IncA/C plasmids and SXT/R391 ICEs (Wozniak and Waldor, 2010; Toleman and Walsh, 2011). Note that the traditional association of highly transmissible elements with plasmids is not necessarily true.

Plasmids are abundant in nature and consistently isolated from microbial communities of different habitats with and without anthropogenic exposure (Coombs, 2009; Sobecky and Hazen, 2009). In fact, contemporary resistance plasmids are based on plasmid backgrounds existing in the pre-antibiotic era (Datta and Hughes, 1983; Hughes and Datta, 1983). Maintenance of plasmids and ICEs in bacterial populations results from both the selfish features that promote acquisition and persistence within bacterial populations ("the parasitic hypothesis") and the beneficial effects they confer to individual bacterial hosts and communities ("the evolvability hypothesis"; Werren, 2011). MGEs are increasingly being considered within this multi-hierarchical model, as clonal-, species-, or community-specific mobile elements (Carattoli, 2009; Garcillán-Barcia et al., 2009; Lim et al., 2010; Shearer et al., 2011; Heuer et al., 2012; Williams et al., 2012; Clewell et al., 2013; Guglielmini et al., 2013). Between and also within these hierarchical levels, plasmids may eventually evolve toward mutualism (Rankin et al., 2011b). Plasmids might assure their permanent linkage with a particular host, bacterial lineage, or multi-specific community by post-segregation killer strategies that cause the death of non-carrying bacterial offspring. This is caused by toxin–antitoxin (TA), restriction-modification (R-M), or clustered regularly interspaced short palindromic repeats (CRISPR) systems, and also probably by "plasmid domestication," which is produced by changes either in the plasmid and host genome that lead to a more stable coexistence (Bouma and Lenski, 1988; Jones, 2010; Marraffini and Sontheimer, 2010; García-Quintanilla and Casadesús, 2011).

The diversity of bacterial communities, the relative population densities of their components, the spatial separation, and nutrient availability greatly influence plasmid host-range, content, and transferability (Coombs, 2009). It is interesting to suggest that the selective processes exerted by antibiotics will modify bacterial diversity and population densities, forcing the coexistence of plasmids and particular hosts, favoring recombination and other processes that lead to plasmid domestication (Boyd et al., 1996). The robustness of interactions between particular plasmids and particular clones is shaped by epistatic processes (Silva et al., 2011; San Millán et al., 2012) mediated by nucleoid-associated transcriptional regulators (Doyle et al., 2007; Yun et al., 2010; Fernández-Alarcón et al., 2011; Humphrey et al., 2012) and the clonal interferences that might result from these interactions (Hughes et al., 2012). A robust interaction is reflected in a non-cost or even negative-cost coexistence, and will tie the fate of plasmid density to the abundance of their specific bacterial hosts, resulting in a necessary "in-host" plasmid evolution linked to "with plasmid" host evolution (Dionisio et al., 2005; Halary et al., 2010).

A major topic in plasmid population biology is the consideration of advantages and inconveniences of plasmid or ICE broad-host-range. It is of note that host-range does not necessarily mean transfer ability to a particular host or long-term maintenance in bacterial populations, but stable replication in a new host (De Gelder et al., 2007; Suzuki et al., 2010). Certainly, broad-host-range conjugative plasmids favor the penetration of adaptive traits as "new" antibiotic resistance genes (Fernández-Alarcón et al., 2011; Sen et al., 2011; Hamprecht et al., 2012; Heuer and Smalla, 2012) and, in turn, antibiotics can favor the abundance of these plasmids promoting transfer and by selection of plasmid containing bacteria (Lang et al., 2012). The frequent observation of different systems of replication (recognizing host primases) in the same plasmid suggests adaptive ways of increasing host-range (Del Solar et al., 1996; Clewell et al., 2013). Globally spread plasmids identified in hospitals, soils, agriculture, and marine habitats have a complex mosaic structure that reflects inter-genomic historical adaptations to phylogenetically distant bacterial hosts (Schlüter et al., 2007; Norberg et al., 2011; Heuer et al., 2012; Partridge and Iredell, 2012). In addition, broad-host-range plasmids lacking transfer systems can be transferred to phylogenetically close or distantly related bacteria by helper conjugation systems located in narrow-host-range plasmids containing a conjugation system (Smorawinska et al., 2012).

Long-term maintenance and dispersion of broad-host-range plasmids in bacterial populations and communities seems to be related with the local availability of hosts (De Gelder et al., 2007), but also with the "social interactions between plasmids" eventually leading to unbearable costs for their hosts (Smith, 2012). Eventually, exclusion mechanisms between plasmids (one plasmid excludes the incoming one) might be softened by inter-plasmid recombination that might result in hybrids able to evade exclusion. CRISPR is a genetic interference system by which bacteria with CRISPR regions carrying DNA copies of previously encountered plasmids can abort the replication of plasmids with these sequences. Hypothetically that might favor plasmid dispersal among different strains, providing a weak selective advantage for the host cell (Levin, 2010), although an increased benefit could be predicted for host coalitions, as genetic exchange communities (GECs; see later). This system also controls phages, but the possible populational interactions (competition–cooperation) between phages and plasmids have scarcely been investigated.

Antibiotic exposure might have increased the absolute number of plasmids with resistance determinants in bacterial populations due to the selection of clones harboring them. The possibility that broad-host conjugative plasmids have been submitted to a more effective enrichment than narrow-host plasmids (because of selection in multiple hosts and environmental compartments) poses an interesting question. Eventually, the biological cost of resistance plasmids for the host could be compensated by higher transmission (Garcillán-Barcia et al., 2011). As Stokes and Gillings pointed out, an increase in the general *tempo* of resistance genes dissemination is highly probable, due to selection of cells with inherently higher rates of lateral transfer (Stokes and Gillings, 2011).

Finally, we can consider bacteriophages as mobile mediators of inter-bacterial transfer of resistance genes. Also in this case antibiotics might modulate phage–bacteria population dynamics by processes as "phage-antibiotic synergy," a non-SOS mechanism of virulent phage induction caused by exposure to sub-inhibitory concentrations of beta-lactams (Comeau et al., 2007; Allen et al., 2011; Looft et al., 2012). Antibiotics promote the number of phages and pro-phages linked to antibiotic resistance platforms, favoring dissemination of these platforms, and consequently amplifying the dissemination of resistance and virulence genes (Allen et al., 2011).

ANTIBIOTICS AND POPULATIONS OF BACTERIAL CLONES AND SPECIES

Bacterial clones are constituted by isolates that have a close common phylogenetic origin. High-risk clones are defined as clones with an enhanced ability to colonize, spread, and persist in a variety of niches, which acquire adaptive traits that increase pathogenicity or antibiotic resistance (Baquero and Coque, 2011). They constitute the main vehicles dispersing antibiotic resistance at a global scale (Willems et al., 2011; Woodford et al., 2011). Examples of these high-risk clones are *E. coli* ST131 (phylogroup B2), ST155 and ST393 (phylogroup B1), or ST69, ST405, and ST648 (phylogroup D); *K. pneumoniae* ST258, ST14 or ST37; *Enterococcus faecium*, ST18, ST17, ST78; *Enterococcus faecalis* ST6; *S. aureus*, ST45, ST5, ST8, ST30, or ST22. A number of these clones were ancient lineages, well-adapted to colonization and transmission between particular hosts, that acquired antibiotic resistance and consequently enhanced capabilities of dispersal (McBride et al., 2007; Brisse et al., 2009; Chambers and Deleo, 2009; Willems et al., 2012). Multiplication and spread of highly successful clones implies multiplication and spread of all the antibiotic resistance genes and platforms they contain, increasing their absolute numbers. In fact, it is not unusual that a single successful clone might contribute to the spread of different plasmids, genetic platforms, and resistance genes, both in Gram-positives (Chambers and Deleo, 2009) and in Gram-negatives (Carattoli, 2009; Andrade et al., 2011; Woodford et al., 2011; Novais et al., 2012a; Partridge and Iredell, 2012). Such multi-lateral collaboration probably contributes to the local ecological success of variants arising in these clones, leading to a clonal diversification (clonalization) which assures a long-term permanence in complex adaptive landscapes, following the "never put all the eggs in the same basket" principle (Wiedenbeck and Cohan, 2011; de Regt et al., 2012; Freitas et al., 2013). Focusing only on mutational evolution, it has been suggested that there is an acceleration of emergence of bacterial antibiotic resistance in connected microenvironments (Zhang et al., 2011; Hallatschek, 2012) but this might also occur in the case of gene flow.

Local clonalization might result in a restricted gene flow among resulting subpopulations (Willems et al., 2012). However, recombinational events might spare those regions required for adaptation to local microniches, assuring divergence, and be maintained for other regions (ecological speciation with-gene-flow; Via, 2012). The increased recovery of isolates belonging to high-risk clonal complexes of important human pathogens as *E. coli, S. aureus, E. faecalis*, or *E. faecium,* that cause both human infections and mucosal colonization, and even the expansion of these clones to novel hosts is most probably related with the acquisition

of antibiotic resistance genes (Hidron et al., 2008; Baquero, 2012; Novais et al., 2012a, 2013)

An interesting question is if the selection of particular clones because of antibiotic resistance might decrease the overall clonal diversity as might be inferred from recent studies (Ghosh et al., 2011). Actually, that might be compensated by clonal diversification, in a "*ex pluribus unum/ex unibus plurum*" evolutionary dynamics (Baquero, 2011). It is easy to conclude that any outbreak produced by antibiotic resistant bacteria will locally enrich the involved evolutionary units, facilitating further events of antibiotic resistance development and possibly transmission (Chambers and Deleo, 2009; Freitas et al., 2013).

ANTIBIOTICS AND POPULATIONS OF BACTERIAL COMMUNITIES

Imbedded into the high complexity of the microbiomes, of humans and animals, in the soil or in the water sediments, it is possible to recognize "clubs" of bacterial clones and species where genes and genetic platforms circulate via lateral transfer, the GECs. In a very restrictive manner, Skippington and Ragan (2011) have recently defined a GEC as a group of organisms (entities) in which each entity has over time both donated genetic material to, and received genetic material from every other entity in that GEC, via a path of lateral gene transfer. What do the members of such clubs have in common? Network modeling and co-occurrence statistical approaches indicate that lifestyle and shared environments, functional complementarities, and most probably, continued physical clustering (granularity) determine the size and connectivity of GECs (Freilich et al., 2010; Smillie et al., 2011; Faust and Raes, 2012; Faust et al., 2012). In many cases, this also means a closed shared phylogeny (Skippington and Ragan, 2012). In fact, GECs members are linked not only by genetic exchanges, but also by metabolic and functional cooperation, providing a certain ecological compartmentalization inside particular microbial megasystems (as intestinal microbiota; Faust and Raes, 2012; Faust et al., 2012). We can consider here some kind of cooperative "niche construction" (Laland et al., 1999; Kylafis and Loreau, 2011). Genetic transfer, particularly considering mixed-granular "surface-associated populations" with a kind of "lattice reciprocity" (Zhong et al., 2010, 2012) might assure a "collective" adaptation of such functional GEC units, increasing relatedness among members and fixing common evolutionary boundaries (Nogueira et al., 2009; Rankin et al., 2011a,b). The development of more studies on the "physics" of genetic transfer, is certainly advisable, for instance, to investigate to what extent genetic transfer can be influenced by mixing movements or the viscosity or fluidity of the surrounding medium, as in the intestinal content (particularly during enteric diseases) or in water bodies, influencing cell-to-cell contacts and therefore GECs integrities (Zhong et al., 2010; Jeffery et al., 2012).

An important topic is the possibility of "multiclonal GECs" as a form of organization of the lifestyle of a particular species or closely phylogenetically related coalitions in changing environments (Skippington and Ragan, 2012), where different subspecific ecotypes exploiting neighbor nano-niches (Wiedenbeck and Cohan, 2011) and taking advantage of a common "public good" are frequently encoded by conjugative elements (Norman et al., 2009; Rankin et al., 2011b). The distribution among GECs members of such plasmid-mediated "public goods" is favored by common characteristics in the consortium, as nearly identical immune phenotypes mediated by CRISPR, or common DNA uptake mechanisms or quorum sensing responses. Plasmid-mediated common benefits will probably lead to GEC-plasmid coevolution (Skippington and Ragan, 2012). Along the same line, addiction-type TA complexes can spread on plasmids, favoring coexistence and/or competition in spatially structured environments (Rankin et al., 2012) highlighting the role of kin effects in GECs selection (taking "kin" in a wider sense than just intraspecific ties). It is of note, however, that possibly most organisms and environments might act as conduits for resistance gene flow (Stokes and Gillings, 2011), acting as "sources" from where resistance genes are directed to GECs, acting as "sinks," according to the Perron et al. (2007) metaphor. The possibility of "go-between" organisms traveling from GECs of different microbiotic systems (humans, animals, rhizosphere, and water sediments) should be taken into consideration, as they might contribute to the inter-environmental globalization of antibiotic resistance genes. The existence of "ubiquitous" organisms or species able to transit in different environments has been suggested (Fondi and Fani, 2010; Freilich et al., 2010; Tamames et al., 2010). Candidates for efficient "go-between" organisms are groups of the same bacterial species but specialized in particular environments, as the case of human or animal-adapted versus environmental *E. coli* clades, where probably only ecological barriers prevent gene flow (Freilich et al., 2010; Luo et al., 2011). Mixing of human or animal derived water effluents into the environment, a practice that is surprisingly perpetuated even in modern societies will facilitate conduits for resistance gene flow (Baquero et al., 2008; Czekalski et al., 2012; Lupo et al., 2012). Such flow occurs because of the confluence of human microbiota from different human hosts, different animals and the indigenous environmental microbiota.

Among these GECs, the most relevant for the transmission of antibiotic resistance are those including species from Gammaproteobacteria (as *E. coli*) and Firmicutes (as *Enterococcus*; Antonopoulos et al., 2009; Freilich et al., 2010; Skippington and Ragan, 2011; Faust et al., 2012). These groups of organisms are enriched in the microbiota during antibiotic exposure (Antonopoulos et al., 2009; Sommer et al., 2009). Antibiotic-mediated reduction in number or loss of some species, favors bacterial species able to explore and temporarily exploit empty niches due to short generation times (Allen et al., 2011; Looft and Allen, 2012). Antibiotic exposure will increase the absolute number (overgrowth) of GECs-associated organisms, for instance, by antibiotic exposure of the infant gut (Fouhy et al., 2012; Looft and Allen, 2012). Probably the same might occur in environmental GECs submitted either to antibiotic pollution or sanitation procedures (Baquero et al., 2008). For instance, metagenomic analysis indicates that drinking water chlorination could concentrate populations containing insertion sequences, integrons, and antibiotic resistance plasmids (Shi et al., 2013). These effects will contribute to the dissemination of resistance genes and the genetic platforms in which they are located.

The identification of GECs among members of the "microbial guilds" in the three identified major microbiome "enterotypes" (Arumugam et al., 2011) needs to be investigated. These major enterotypes (clusters of species) should probably be tightly maintained into host populations, and therefore the local spread of antibiotic resistance genes could serve to maintain their integrity in an antibiotic-polluted environment. At the same time, low-abundance enterotype-species, but providing critical functions, could have low possibilities of getting resistance genes in the absence of GECs. Consequently, a deep and permanent contamination by antibiotic resistance genes of the normal microbiota of humans and animals is a reasonable possibility. Complex microbiota of humans and animals are reproducible systems, not only along time in the same individual, but also across individuals. These systems are frequently based in a microbiotic "core" composed by organisms belonging to a relatively small number of phylogroups, and probably metabolically linked with the host (Dethlefsen et al., 2006; Ley et al., 2006; Marchesi, 2011). Newborns have a sterile gut, but the human microbiota is "reproduced" with a relatively low number of variations on each of them (Baquero and Nombela, 2012; Vallès et al., 2012). Possibly there is also an "epidemiology of bacterial consortia" even in hospitals, which remains to be investigated. Exposure to antimicrobial agents might affect the frequency and absolute number of GECs; in a number of cases, antibiotic resistance might contribute to the temporal stability and resilience of microbiomes in an antibiotic-polluted environment (Allison and Martiny, 2008; Antonopoulos et al., 2009). Of course that occurs at the expenses of maintenance and spread of the full range of antibiotic resistance evolutionary units.

Finally, we cannot discard individual variations in the microbiotic communities caused by diet, host genetics, particular illnesses, inflammation, or infectious agents including viruses and parasites which might lead to microbial communities more prone to capture and propagate antibiotic resistance (Marchesi, 2010; Claesson et al., 2012; Looft and Allen, 2012). For instance, a high-fat diet determines the composition of the murine gut microbiome independently of obesity, with an increase of Proteobacteria and Firmicutes, heavily involved in resistance gene mobilization (Hildebrandt et al., 2009; Tagliabue and Elli, 2012). In several microbiota communities studied in the elderly, the proportion of phylum Proteobacteria, very active in the mobilization of antibiotic resistance genes and vehicles, was ten times higher than average (20 versus 2%; Claesson et al., 2011). *E. coli* numbers are higher in the microbiota of women with excessive weight gain than in women with normal weight gain during pregnancy (Santacruz et al., 2010).

A number of surgical interventions (as surgery for morbid obesity) increases Proteobacteria even in a higher proportion (50 times increase; Li et al., 2011; Graessler et al., 2012). Unfortunately, these populational microbiotic shifts favoring the active populations and communities contributing to the emergence, dispersal and maintenance of antibiotic resistance might also occur as a consequence of undernutrition (10 times increase in Proteobacteria, 46 versus 5% in healthy children in Bangladesh; Monira et al., 2011). Possibly the deleterious effect of antibiotics in promoting antibiotic resistance will be significantly increased. Finally, it could be considered, under certain circumstances, as during the colonization of the neonatal intestinal tract, that rapidly growing populations might be more prone to the dissemination of antibiotic resistance. Also the unexpected possibility of resistance gene exchange between Actinobacteria (*Bifidobacterium* belongs to this group!) and Gammaproteobacteria has been recently shown under the same conditions (Tamminen et al., 2012).

ANTIBIOTICS IN THE ANTHROPOCENE: EFFECTS ON GLOBAL ECOLOGY AND EVOLUTION

Evolution is a natural trend of complex systems, and might be accelerated by changing and stressful conditions. The Anthropocene is the current human-dominated geological epoch where nature is changed and stressed by the action of humans (Zalasiewicz et al., 2011; Biermann et al., 2012). Industrial antibiotics are a paradigmatic example of substances exerting a powerful effect of anthropogenic origin on the bacterial communities of the microbiosphere. Not only most of these substances are unspecifically killing bacterial organisms, and selecting for resistance, but directly influence the mechanisms of genetic variation (mutation, recombination, transposition, modularization, gene transfer; Baquero, 2009; Gillings and Stokes, 2012).

Such effects on microorganisms will be further enhanced by a diversity of other anthropogenic effects as the release of biopharmaceuticals, biocides, heavy metals, industrial and agricultural residues, and plastic materials or changes in the environmental conditions. The mixing of bacterial populations (human organisms with other human, animal, or environmental organisms), that makes the emergence and spread of resistance possible is also favored by poor sanitation, facilitating contact of human or animal sewage with the soil. Some of these effects might escalate with other anthropogenic effects as destruction of diversity in food animals (Baquero, 2012) or even global warming (Baquero et al., 2008; Baquero, 2009; Balbus et al., 2013). The fight against antibiotic resistance should focus not only on acting on its appropriate usage in human and veterinary medicine, but by considering possible initiatives at ecological and evolutionary levels, as eco-evo drugs and strategies (Baquero et al., 2011) in accordance with the environmental distribution of bacterial organisms (Tamames et al., 2010), in the scope of progressing toward a protective and restorative planet medicine (Baquero, 2009).

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Antibiotics as selectors and accelerators of diversity in the mechanisms of resistance: from the resistome to genetic plasticity in the β-lactamases world

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Antibiotics and antibiotic resistance determinants, natural molecules closely related to bacterial physiology and consistent with an ancient origin, are not only present in antibiotic-producing bacteria. Throughput sequencing technologies have revealed an unexpected reservoir of antibiotic resistance in the environment. These data suggest that co-evolution between antibiotic and antibiotic resistance genes has occurred since the beginning of time. This evolutionary race has probably been slow because of highly regulated processes and low antibiotic concentrations. Therefore to understand this global problem, a new variable must be introduced, that the antibiotic resistance is a natural event, inherent to life. However, the industrial production of natural and synthetic antibiotics has dramatically accelerated this race, selecting some of the many resistance genes present in nature and contributing to their diversification. One of the best models available to understand the biological impact of selection and diversification are β-lactamases. They constitute the most widespread mechanism of resistance, at least among pathogenic bacteria, with more than 1000 enzymes identified in the literature. In the last years, there has been growing concern about the description, spread, and diversification of β-lactamases with carbapenemase activity and AmpC-type in plasmids. Phylogenies of these enzymes help the understanding of the evolutionary forces driving their selection. Moreover, understanding the adaptive potential of β-lactamases contribute to exploration the evolutionary antagonists trajectories through the design of more efficient synthetic molecules. In this review, we attempt to analyze the antibiotic resistance problem from intrinsic and environmental resistomes to the adaptive potential of resistance genes and the driving forces involved in their diversification, in order to provide a global perspective of the resistance problem.

Keywords: environmental resistome, intrinsic resistome, plasticity, β-lactamase

INTRODUCTION

Nowadays and thanks to the high-throughput sequencing tools and bioinformatics software, knowledge on high bacterial diversity in bacterial communities (metagenome) is increasing. A huge diversity of resistance mechanisms to practically all antibiotic families has been found in both antibiotic- and non-antibiotic-producing bacteria (D'Costa et al., 2006; Bhullar et al., 2012). Three types of resistome can be defined: intrinsic, environmental, and unknown (Fajardo et al., 2008; Martínez, 2008; Dantas and Sommer, 2012). In the intrinsic resistome or pre-resistome, the antibiotic resistant elements belong to bacterial metabolic networks, reflecting their role in microbial physiology. They might be coupled to signaling molecules (antibiotics) facilitating the co-selection of antibiotics and antibiotic resistance genes in a constant arms-race over a long time (Fajardo and Martínez, 2008). So today the intrinsic resistome is a wider concept and probably universal to the bacterial world. The description of the intrinsic resistome has expanded our knowledge about potential new resistance mechanisms (Dantas et al., 2008; Toleman et al., 2012). They could become true antibiotic resistance genes if appropriate driving forces were exerted. Moreover, the potential adaptiveness of these pre-resistome genes can be accelerated if, by chance, they are transferred to new genetic contexts (exaptation; **Figure 1**), where these genes may evolve toward more efficient enzymes without having a physiological role (Baquero et al., 2009; Gullberg et al., 2011). As a consequence, this silent and non-predictive resistome (unknown resistome) is ready to be selected (Baquero, 2012).

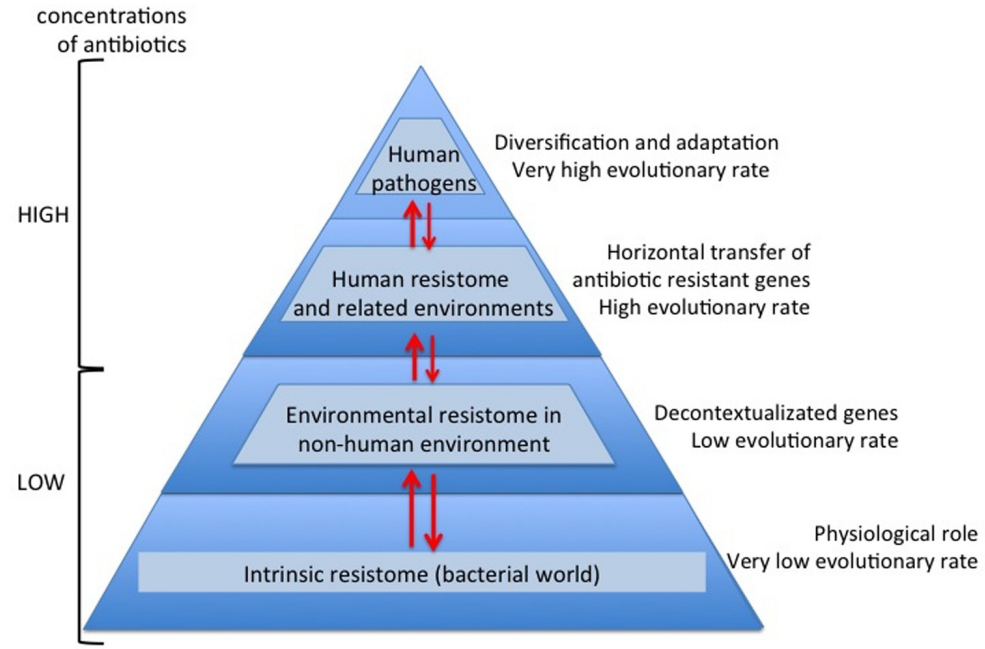

FIGURE 1 | Overview evolutionary processes of the antibiotic resistance. During millions of years antibiotics and antibiotic resistance genes have co-evolved slowly. In this long period the first transition was the acquisition of pre-resistance genes by different bacteria (exaptation). This genetic transference allowed the evolution toward true and more efficient antibiotic resistance genes. However, the great evolutionary transition was the discovery, mass production and consumption of antibiotics. Antibiotics accelerated dramatically the diversification of resistance genes and selection for reaching extraordinary efficient variants.

Efforts to understand the spread of antibiotic resistance and find previously undescribed antibiotic resistance mechanisms in non-human environments have yielded a more complex view of the antibiotic resistance problem (D'Costa et al., 2006; Dantas et al., 2008; Bhullar et al., 2012). Usually, the bacterial population-nutrient concentration ratio is low in the environment and a model of competitive interactions among microorganisms is established. Microbial competitors are able to produce high levels of natural antibiotics that become toxic compounds killing non-producer competitor strains in order to access the limited nutrients. However, antibiotic susceptible strains were able to develop strategies conferring resistance to these antibiotics, constituting the environmental resistome, starting adaptation, and co-adaptation cycles between antibiotics and antibiotic-resistant proteins (Martínez, 2009). New sequencing platforms have revealed that our old concept that only the antibiotic-producing bacteria were carriers of antibiotic resistance mechanisms was a very simple view. The scientific community has gradually begun to understand that antibiotic resistance has a global distribution in nature, even without the presence of humans, and that antibiotics and antibiotic resistance mechanisms have been evolving for millions of years (Wright, 2010).

The capture of antibiotic resistance or pre-resistance genes from intrinsic, environmental, or unknown resistomes is a stochastic, unpredictable phenomenon. Once the determinants of resistance reach the human environments or environments related to human activity, the evolutionary race between antibiotics and antibiotic resistance genes is drastically accelerated, as a consequence of the high concentration of antibiotics (Martínez et al., 2007). Therefore, antibiotics are not only selector agents of mechanisms of resistance; they are also accelerator agents of the evolution of resistance (**Figure 1**). This is a consequence of the huge plasticity observed in antibiotic resistance genes for acquiring a new spectrum of action or more efficient capacities with respect to the original spectrum (Deng et al., 2012). This observation suggests that the diversification force of resistance elements can be as fast and strong as the antibiotic diversification. For instance, the golden age in the commercialization for clinical use of β-lactam antibiotics was between the late 1970s and mid 1980s. Based on dated phylogenetic reconstructions of the most important β-lactamases in the clinical setting, the majority of diversification events occurred recently, even though the enzymes have been present for millions of years (Barlow and Hall, 2002a,b). *In vitro* models with CTX-M β-lactamases suggest that the explosive molecular diversification of these enzymes could only be explained by the simultaneous presence of different extended spectrum cephalosporins (Novais et al., 2010). Similar results were found with TEM enzymes (Salverda et al., 2011), suggesting that both the environmental contamination with different β-lactams and the potential plasticity of these enzymes could have been the main diversifying forces. Traditionally, the problem of antibiotic resistance has been focused as a clinical problem. Obviously, human health is the main reason, but we will never be able to cope with the antibiotic resistance problem if it is only seen as such. In this review, we attempt to analyze the antibiotic resistance problem from new perspectives. From the intrinsic resistome to the potential adaptiveness

of determinants of resistance, from the environmental resistome to the driving forces involved in the diversity of variants related to specific determinants of resistance, as well as to provide a global view of the antibiotic resistance problem (Davies, 2007; Martínez et al., 2009).

INTRINSIC RESISTANCE OR INTRINSIC RESISTOME

In the EUCAST expert rules on antimicrobial susceptibility testing, "intrinsic resistance" or "inherent resistance" is understood as a feature of all or almost all isolates of a bacterial species and in contrast to the acquired and/or mutational resistance concepts (Leclercq et al., 2011). From a microbiological point of view, intrinsic resistance can be a result of: (i) inherent difficulties for the antibiotic to reach its corresponding target due to impaired permeability of the bacterial envelope or efficient drug export systems, the so-called multi-drug resistance (MDR) efflux pumps; (ii) the absence of antimicrobial target(s) or presence of targets with low affinity; or even (iii) the presence of a mechanism that inhibits or destroys the antibiotics (enzymes that neutralize antibiotics in the cytoplasm or periplasmic space). Some examples of these mechanisms are included in **Table 1**. Nevertheless, all microorganisms contain efflux pumps involved in bacterial physiology, which can participate in resistance to different extents, although the clinical consequences might be of minor importance unless coupled with other resistance mechanisms (Li et al., 1994a,b; Piddock, 2006; Martínez, 2012; Nessar et al., 2012). Often, bacteria combine different mechanisms affecting several antimicrobial drugs. Conventionally, the intrinsic resistome has been defined as the set of chromosomal genes that are involved in intrinsic resistance and whose presence in strains of a bacterial species is independent of previous antibiotic exposure and is not due to horizontal gene transfer (Martínez, 2012).

Nowadays, the "intrinsic resistome" is a wider concept than intrinsic resistance and includes genetic elements normally belonging to bacterial metabolic networks that can eventually participate in resistance to antimicrobial agents. Pre-resistance genes constituting the intrinsic resistome has two main characteristics: firstly, they confer low level of resistance which is also known as "pre-resistome" (Fajardo et al., 2008); secondly, the different resistance mechanisms are expressed at low levels, but their expression can increase due to the involvement of regulatory genes or as a consequence of mutational events, the so called "silent resistome" (Dantas and Sommer, 2012). Global transcriptome analyses of microorganisms grown in the presence of β-lactams induce the expression of *ampC* (Bagge et al., 2004). This chromosomal gene, present in the genome of *Pseudomonas aeruginosa* and of many Enterobacteriaceae for several hundred millions of years, plays a physiological role in the normal course of peptidoglycan synthesis, remodeling and recycling the bacterial envelope (Jacobs et al., 1994; Henderson et al., 1997).

For years recognition of the intrinsic resistome has not been an easy task. This will not be the case in the coming years as the implementation of whole genome sequencing strategies and bioinformatic tools for genome comparisons and knockout procedures will increase the recognition of determinants that might be involved in resistance phenotypes, even if they are very lowly expressed (Didelot et al., 2012; Huang et al., 2012; Schmieder and Edwards, 2012). The increased expression of these systems increases resistance levels (increased MIC values) whereas their absence increases bacterial susceptibility (decreased MIC values; Alekshun and Levy, 2007).

Hence, in bacterial resistance the differentiation between *intrinsic resistome* and *intrinsic resistance* is a thin line, but low-level resistance can be associated at a certain point to *intrinsic resistome* and high-level resistance to *intrinsic resistance* (Leclercq et al., 2011).

ANTIBIOTICS AND ANTIBIOTIC RESISTANCE GENES HAVE ANCIENT ORIGINS

During the 1970s, it was shown that resistance genes related to aminoglycoside modifying enzymes (AMEs) in clinical bacteria had their origin in common soil bacteria belonging to *Actinomycetes*, which also produce AMEs (Benveniste and Davies, 1973). However, metagenome studies from different sources have questioned the "hypothesis of the antibiotic producers" because antibiotic resistance genes have been found in the microbiome of ancient isolated environments dating back a million years (D'Costa et al., 2006; Allen et al., 2010; Torres-Cortés et al., 2011; Bhullar et al., 2012). The functional role of antibiotic resistance genes in antibiotic-producing bacteria seems clear: they need to protect themselves from the activity of their own antimicrobials; however, in non-producers their role is less evident. Some explanations have been suggested. First, antibiotic resistance genes have or have had a physiological role in bacteria (see below). Second, antimicrobial agents are more prevalent than suspected. Analyses of bacterial genome sequences suggest that only 10% of the natural antibiotics have been discovered and probably only 1% of antimicrobial molecules from producers are known (Fischbach, 2009), possibly because they are natural products of bacterial physiology (Osbourn, 2010). More probably both scenarios are true and non-exclusionary. The complex network of physiological interactions that constitute the intrinsic resistome is coupled to small natural molecules, such as antibiotics, which might have a role as signaling molecules. According to this concept, antibiotics and

Table 1 | Different examples of intrinsic resistance with clinical relevance.

Intrinsic resistance mechanisms class	Resistance mechanism	Antibiotics affected	Microorganisms
Inadequate target	PBP5 mutations	Cephalosporins	*Enterococcus faecium*
Inactivating process	L1 beta-lactamase	Carbapenems	*Stenotrophomonas maltophilia*
Impaired permeability	Impermeable cell barrier	Vancomycin	Enterobacteriaceae

antibiotic resistance genes have evolved during millions of years by forming interactions in coupled bacterial-specific networks, both by the same and different species (Arifuzzaman et al., 2006). For instance, different *Staphylococcus aureus* strains synthesize peptides that are recognized as signals by strains belonging to the same group and are competitive inhibitors against other *S. aureus* strains belonging to different lineages (Ji et al., 1997).

An accepted hypothesis is that these low molecular weight natural products (antibiotics) could originally be signaling molecules that help shaping the structure of microbial communities (Yim et al., 2007; Linares et al., 2006; Fajardo and Martínez, 2008). Even quinolone compound derivatives, which are produced by a variety of plants and microorganisms, have been found to act as quorum-sensing signal molecules, controlling the expression of virulence genes as a function of cell population density. This has been particularly investigated in *P. aeruginosa*. In this species, these compounds play multiple roles as membrane-interacting compounds, inhibitors of cytochrome complexes and iron chelators, as well as in the regulation of their biosynthesis and their integration into the intricate quorum-sensing regulatory networks governing virulence and secondary metabolite gene expression (Heeb et al., 2011). This could also be the case of other antibiotics, including small peptide molecules that might have a potential role in complex bacterial communities (microbiomes; Belda-Ferre et al., 2012). Therefore, the production of antibiotics is under strict genetic control. In resource-limited environments or when bacterial cells reach the stationary phase, the production of microbial secondary metabolites, such as microcins, is increased in order to yield a survival advantage to the producing bacteria (Romero et al., 2011). Microcins are DNA-damaging agents and, in consequence, the SOS regulon is induced and the chromosomal gene encoding the physiological inhibitor DNA gyrase, *gyrI*, is over-expressed (Baquero et al., 1995). This case is one of the examples showing that antibiotics and antibiotic resistance genes are natural products in constant and ancestral co-evolution (Chatterji and Nagaraja, 2002). Another interesting example is that of β-lactamases, which are structurally similar to PBPs (penicillin-binding proteins), the target of β-lactam antibiotics, the enzymes involved in the metabolism of peptidoglycan (Massova and Mobashery, 1998). Some PBPs such as PBP5 have weak β-lactamase activity conferring a low β-lactam resistance phenotype (Sarkar et al., 2011). In conclusion, antibiotics and also antibiotic resistance genes might have a dual functional and ecological role. At low concentrations antibiotics are signaling systems and at high concentrations they are weapons (Fajardo and Martínez, 2008).

PHYSIOLOGICAL ROLE OF THE INTRINSIC RESISTOME ELEMENTS AND THE UNKNOWN OR SILENT RESISTOME

It has been anticipated that the intrinsic resistome elements have a physiological role in bacteria other than conferring resistance to antibiotics currently used in the clinical practice (Wright, 2007, 2010). A recent review addressing the importance of *Mycobacterium abscessus*, a rapidly growing bacteria involved in soft-tissue infections and chronic pulmonary diseases, showed the presence of a high number of resistance mechanisms responsible for natural resistance in this species, including efflux pumps, antibiotic-modifying enzymes, and target-modifying enzymes (Nessar et al., 2012). Most of these genes have physiological functions but express resistance in the presence of antibiotics, again suggesting that antibiotic resistance genes are the result of specific adaptive responses in genes with previous physiological roles.

The idea that intrinsic resistance is a consequence of the global bacterial physiology was later demonstrated with the study of the intrinsic resistomes of *P. aeruginosa* (Fajardo et al., 2008) and *Escherichia coli* (Tamae et al., 2008). *P. aeruginosa* is thought to be intrinsically resistant to several antimicrobial agents mainly due to a reduced cellular permeability and the activity of MDR efflux pumps. Fajardo et al. (2008) performed a transposon-tagging experiment to determine the elements involved in the intrinsic resistance of *P. aeruginosa*. They demonstrated that ∼2% of the *P. aeruginosa* genome is involved in the altered susceptibility against different categories of antibiotics, but surprisingly only one gene had been annotated as an antibiotic resistance gene (Fajardo et al., 2008). Many of the genes detected as related to antibiotic resistance are involved in basic bacterial metabolism. However, their phenotypic effect is weak; therefore, the development of a real intrinsic resistance phenotype requires a complex assemblage of mutations, such as those previously commented in efflux pumps.

Those genes with a physiological function in the bacteria representing genes that do not confer clinical resistance to their native host, but are capable of expressing resistance when up-regulated or expressed in other hosts (exaptation) can eventually be involved in resistance will be part of the so-called "unknown resistome." Elements from the intrinsic resistome and the unknown resistome have been stressed as potential targets for the design of new interventions to prevent antimicrobial resistance (Martínez, 2012).

THE ENVIRONMENTAL RESISTOME

Although the origin of antibiotic resistance was mysterious in the past, it has become evident over the last decade that environmental bacteria were highly resistant to antibiotics (Wright, 2010). In fact this was discovered 40 years ago by Benveniste and Davies (1973) who found that AMEs from various species of the genus *Streptomyces* were similar to those found in clinical bacteria. Similarly, resistance to vancomycin, first reported in 1988 in *Enterococcus faecium* clinical isolates in Europe (Leclercq et al., 1988), was later found to be associated with glycopeptide antibiotic-producing *Actinomycetes* from soil (Marshall et al., 1997, 1998) as well as in non-antibiotic-producing bacteria of the genera *Paenibacillus* and *Rhodococcus* from soil (Guardabassi et al., 2004). More recently it has been demonstrated that several antibiotic resistance genes (genes conferring resistance to β-lactams, aminoglycosides, tetracyclines, sulfonamides, and phenicoles) have been exchanged between environmental bacteria and clinical pathogens on the basis of perfect nucleotide sequence identities of these genes with those from diverse human pathogens (Forsberg et al., 2012). In 2010, Knapp et al. (2010) quantitatively analyzed the abundance of 18 antibiotic resistance genes from five long-term-soil extracted DNAs from different areas in The Netherlands covering the years 1940 to 2008. They found that antibiotic resistance genes from all classes of antibiotics have increased since 1940, especially for tetracycline and β-lactam resistance encoding genes that have a higher abundance in the twenty-first century when compared with the 1970s, suggesting that levels of antibiotic resistance genes

in the environment are now high and increasing (Knapp et al., 2010). Although resistant organisms in the environment may be the result of contamination by the recent use and misuse of antibiotics by humans, this previous dogma is now seriously disputed thanks again to the advances in new high throughput sequencing technologies (Rolain et al., 2012).

Aquatic environments are also a source for antibiotic resistance genes. The bacteria and/or antibiotic resistance genes could be transmitted directly or indirectly from these environments to humans (Zhang et al., 2009) as result of contamination by the recent use of antibiotics in agriculture and aquaculture selecting these intrinsic resistance genes (Baquero et al., 2008; Zhang et al., 2009; Wright, 2010). Many different antibiotic resistance genes from different classes of antibiotics (tetracyclines, aminoglycosides, macrolides, chloramphenicol, vancomycin, sulfonamides, and β-lactams) have been detected in different water environments worldwide using different molecular techniques as reviewed recently (Baquero et al., 2008; Zhang et al., 2009). The recent emergence and spread of carbapenemase encoding genes in Gram-negative bacteria seems to have originated from bacterial species in water environments (Lupo et al., 2012). For example, partial OXA-23 like carbapenemase sequences have been detected in groundwater samples from the Katmandu Valley of Nepal (Tanaka et al., 2012) that were 100% similar to those of sequences found in human head lice from Senegal (Kempf et al., 2012), suggesting that arthropods may also be a source of antibiotic resistance genes in human pathogens. Recently, MDR *E. coli* and *Klebsiella* spp. with extended spectrum β-lactamase (ESBLs) have been cultured from municipal wastewater treatment plant effluents in the Czech Republic (Dolejska et al., 2011). Similarly, several bacterial species including human pathogens carrying NDM-1, recently discovered (Yong et al., 2009) and spreading worldwide (Rolain et al., 2010), have been detected in tap and drinking water as well as drain and sewage in India (Walsh et al., 2011; Shahid et al., 2012) and in seepage water (rivers, lakes, and water pools in streets) from Vietnam (Isozumi et al., 2012). These data implicate contaminated aquatic environments, as a likely site for the exchange of antibiotic resistance genes between the clinic and the environment (Baquero et al., 2008).

Wild animals and pets, especially their gut microbiota, may also be a source for antibiotic resistance genes that come in contact with humans (Allen et al., 2010). Pets, cats and dogs in particular, may be a source for antibiotic resistance genes that could be transmitted to humans and the environment and *vice versa* (Guardabassi et al., 2004). It has been shown that resistance to antibiotics in bacteria recovered from pets has increased markedly over the last decade, especially the emergence of MDR *Acinetobacter baumannii*, *E. coli*, *Salmonella enterica*, *Staphylococcus intermedius*, or methicillin-resistant *S. aureus* that could be a risk for transmission to humans (Lloyd, 2007). Gilliver et al. (1999) reported more than 10 years ago that antibiotic resistance was highly prevalent even in wild rodents that were believed not to be exposed to antibiotics. Similarly, vancomycin-resistant enterococci carrying the *vanA* gene have been isolated from feces of wild mammals in the United Kingdom (Mallon et al., 2002), OXA-23 carbapenemase encoding gene in *Acinetobacter* species from the gut microbiota of cattle in France (Poirel et al., 2012), or MDR *E. coli* in European wild boars (Poeta et al., 2009; Literak et al., 2010), pigs, and rodents (Literak et al., 2009). Wild birds may also be a source for antibiotic resistance genes and their dissemination by migratory birds, as recently exemplified using functional metagenomics of a huge diversity of antibiotic resistance genes in fecal samples from a breeding colony of gulls in the United States (Martiny et al., 2011) or ESBL *E. coli* in great cormorans and mallards in Europe (Tausova et al., 2012). Finally, it has been recently shown that feces from urban pigeons in Brazil contain MDR enterococci (da Silva et al., 2012). Similarly, MDR enterococci including *Enterococcus faecalis* and *Enterococcus gallinarum* isolates resistant to vancomycin and MDR *E. coli* have been recovered from feces of feral pigeons in the Czech Republic (Radimersky et al., 2010), suggesting that pigeons should be considered a risk species that may contribute to the spread of resistance in the urban environment.

In recent years, new technological approaches, especially functional metagenomics, have led to the characterization and discovery of many unknown antibiotic resistance genes, the so-called resistome, i.e., all antibiotic resistance genes at a global scale in any bacteria (Wright, 2007). Functional metagenomic approaches have been developed and used to decipher the resistome in soil samples by Handelsman (2004) in 2004 that led to the discovery of new antibiotic resistance genes such as AMEs and β-lactamases. The first study of the extent of this resistome was reported by D'Costa et al. (2006) from bacteria isolated from soils showing that many environmental bacteria were MDR and that half of the antibiotic resistance genes discovered were unknown. Moreover, novel mechanisms of resistance involving the inactivation of telithromycin and daptomycin, two antibiotics approved in the last decade were discovered thanks to this approach (D'Costa et al., 2006). Later, Dantas et al. (2008) confirmed the presence of bacteria from soils intrinsically resistant to many different classes of antibiotics.

Nowadays, there is evidence that these antibiotic resistance genes have an ancient origin as demonstrated by the recent discovery using metagenomic analysis of genes conferring resistance to β-lactams, tetracyclines, and glycopeptides in permafrost sediments dating from 30,000 years ago (D'Costa et al., 2011). Similarly, MDR bacteria have been recently cultured from soil samples of an over 4 million year old cave in New Mexico (Bhullar et al., 2012), revealing an unexpected reservoir of antibiotic resistance genes in the environment. Although environmental reservoirs are well known as a possible source of antibiotic resistance genes detected in human pathogens, reports of antibiotic resistance genes from environmental bacteria with a high level of sequence similarity to those from human pathogens previously reported are limited. CTX-M-8 β-lactamase in environmental bacteria of the genus *Kluyvera* (Poirel et al., 2002) or *qnrA*-like genes from marine and freshwater bacteria of the genus *Shewanella* (Poirel et al., 2005) are examples of antibiotic resistance transferred from environmental bacteria to human pathogens. But, the environmental resistome is not only a huge reservoir of antibiotic resistance genes; it is also a generator of new mechanisms of resistance. The finding of a new bifunctional β-lactamase in a remote Alaskan soil sample (Allen et al., 2009) or the chimeric origin of the New Delhi metallo-β-lactamase NDM as the consequence of a recombination event between AME and β-lactamase (Toleman et al., 2012)

are good examples of new antibiotic resistance arising in the environment.

In summary, antibiotic resistance is a natural and ancient phenomenon and its recent increase of antibiotic resistance among pathogenic bacteria has been the result of the ancient and recent mobilization of resistance genes from these environmental and animal reservoirs to human pathogens. Nevertheless, in spite of the efforts to clearly identify transfer events from environmental to pathogenic strains, only a few examples have been documented. The paucity of genetic evidence to date this transference is more likely the consequence of under sampling rather than that these exchanges have not occurred. Our knowledge of the environmental resistome remains low and future studies should be focused on discovery of these new antibiotic resistance genes for the better understanding of the magnitude of this phenomenon in environmental reservoirs and its potential impact in human pathogens. Another reason for the few cases documented so far could be related to the complex interplay between resistome genotypes and resistance phenotypes, which could be context-depend. Only some specific genetic combinations of resistance genes, plasmids, strains, and microbial community can be successful. Therefore, in the absence of experimental evidence the spectrum of action will be difficult to assess (Dantas and Sommer, 2012). The high prevalence and diversity of both antibiotic resistance genes and bacterial species in the environment make these ecosystems an opportunity to decipher the mechanisms of transfer and usage of these genes in bacteria living in a sympatric lifestyle which is a key component for bacterial evolution (Diene et al., 2012). We need to understand the magnitude of this phenomenon in the future in order to be able to define new strategies to face the problem of antibiotic resistance globally and not only from a human medical viewpoint.

MOBILIZATION AND EXPRESSION OF INTRINSIC RESISTOME ELEMENTS

Comparisons of soil microbiota with clinical pathogens have provided evidence of an ancient and recent exchange of antibiotic resistance genes (β-lactams, aminoglycosides, amphenicols, sulfonamides, and tetracyclines) as well as the multiple mobilizations of sequences between these communities. They demonstrate not only evidence of lateral exchange but also a mechanism for the dissemination of antibiotic resistance (Forsberg et al., 2012). If the assumption that the intrinsic resistance has an ancient origin in environmental genes, then it can also be accepted that these genes are transmitted to human pathogenic bacteria. Admittedly these genes could be mobilized from their ancestors. Among these intrinsic resistance genes, β-lactamases genes (*bla* genes) are considered one of the best examples and are also discussed in this review.

As previously commented, amino acid sequence analysis of PBPs and β-lactamases point to a common origin of these proteins (Bush, 1997; Massova and Mobashery, 1998). Nevertheless, different ancestors have been recognized and different evolutionary trajectories might have occurred (Cantón, 2007). The analysis of β-lactamase phylogeny is not congruent with the phylogenetic history of its carriers, as horizontal gene transfer, frequently produced over time, might have interfered in the evolutionary process of β-lactamases. In some cases, such as class D (OXA type) enzymes, they are present both in Gram-positive and Gram-negative bacteria whereas Class C enzymes are only present in Gram-negative organisms (unlike *Mycobacterium smegmatis*), reflecting different evolutionary trajectories. Along these trajectories, β-lactamases have had to undergo structural alterations to become efficient as β-lactam-hydrolyzing enzymes, avoiding the interaction with peptidoglycan (or peptidoglycan precursors), which are the substrates of PBPs. It has been hypothesized that this gives an advantage to β-lactamase-producing bacteria, particularly in soil communities, where natural β-lactam-producing bacteria might be normally present (Bush, 1997).

One of the most studied β-lactamases is the Class A enzymes. Within this group the most widely distributed enzyme is TEM-1, which was first described in 1963 (Datta and Kontomichalou, 1965). The *bla* gene encoding this enzyme is carried in a transposable element (Tn3) that is widely present in plasmids of different incompatibility groups (Hedges and Jacob, 1974). Despite its widespread presence all over the world and in many different pathogenic bacteria, including among others all Enterobacteriaceae, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and *P. aeruginosa*, its ancestor remains undetermined. This is not the case of the CTX-M enzymes, which were recognized as plasmid-mediated enzymes hydrolyzing extended-spectrum cephalosporins (i.e., cefotaxime) in 1989 (Bauernfeind et al., 1990), from environmental bacteria of the genus *Kluyvera* (Poirel et al., 2002).

Nowadays, the CTX-M enzymes are widely disseminated all over the world within Enterobacteriaceae (Cantón and Coque, 2006). The corresponding genes, the $bla_{\mathrm{CTX-M}}$, have been found in chromosomal location in different species of the genus *Kluyvera*, which is generally accepted as the origin of these genes (Cantón et al., 2012). In the *Kluyvera* species, cefotaxime resistance has low levels of expression. Mobilization by insertion sequences (IS), such as IS*Ecp1* or IS*CR1*, has resulted in higher expression of resistance in *E. coli*, *K. pneumoniae*, and other clinically relevant Enterobacteriaceae (Poirel et al., 2003; Lartigue et al., 2006; Rodriguez-Martinez et al., 2006).

Similar to Class A enzymes, the mobilization of bla_{OXA} genes has been estimated to have occurred at different times, the first one a hundred million years ago, whereas the last mobilization occurred very recently. This could have been the case of the carbapenem-hydrolyzing β-lactamase OXA-48 and its derivative OXA-181, which differs from the former by four amino acid substitutions, which have been related with *Shewanella xiamenensis* (Potron et al., 2011). This is an environmental species from marine and freshwaters that contains the $bla_{\mathrm{OXA-181}}$ gene. OXA-48-producing Enterobacteriaceae are increasingly recognized worldwide whereas the detection of OXA-181-producing isolates remains scarce (Poirel et al., 2012). Similarly to some of the $bla_{\mathrm{CTX-M}}$ genes, the $bla_{\mathrm{OXA-181}}$ gene was also preceded by its insertion in the element IS*Ecp1*, which might also have been responsible for its mobilization.

Interestingly, other species from *Shewanella* might also play an important role as the origin and reservoirs of resistance determinants. They have been found to harbor not only different β-lactamases genes but also resistance genes affecting other antibiotic

classes such as fluoroquinolones (*qnrA* gene in *Shewanella algae*; Poirel et al., 2005; Rodriguez-Martinez et al., 2005). In the case of β-lactamases, *Shewanella oneidensis* and *Shewanella algae* harbor chromosomal class D β-lactamases genes (bla_{OXA-54} and bla_{OXA-55}, respectively) and *Shewanella livingstonensis* and *Shewanella frigidimarina* harbor chromosomal metallo-β-lactamase genes (bla_{SLB-1} and bla_{SFB-1}, respectively; Poirel et al., 2005). None of these genes has been found yet in plasmids but they could eventually be mobilized to pathogenic bacteria. Unlike these cases, the bla_{OXA-23} gene, whose progenitor has been found in *Acinetobacter radioresistens*, is nowadays widely spread in *A. baumannii* (Walsh, 2008).

All these examples illustrate how the intrinsic resistome can be mobilized from environmental bacteria and expressed at different levels in new hosts. However, it is surprising that despite the huge environmental and intrinsic resistome in environmental and commensal bacteria we can identify transmission events in only a few cases (**Table 2**; Davies, 1997; Davies and Davies, 2010), suggesting the existence of biochemical, metabolic, and genetic constrictions (Dantas and Sommer, 2012). When we are capable of understanding the "global resistome," i.e., the environmental and intrinsic (including silent) resistome, then all the bacterial potential for fighting other bacteria will be known and only then will we be able to fight the antibiotic war on equal terms. Then, the following steps in our understanding of the resistance process will be to define and reveal the adaptive potential of those physiological genes that evolved toward resistance genes.

PHYLOGENY AND EVOLUTION OF β-LACTAMASES

Antibiotic resistance is probably one of the best examples of "evolution in action." The basic mechanisms of resistance appeared early in the evolutionary time-scale and evolved to counter the new defenses put forward by other bacterial species to compete for resources or survival against competitors and predators (Baquero and Blázquez, 1997; Salmond and Welch, 2008; Davies and Davies, 2010). It has been commented several times throughout this review that antibiotic resistance genes were ancient elements with a physiological role as components of complex networks. The decontextualization of these genes from their original background (exaptation) allowed a slightly faster evolutionary process toward a real antibiotic resistance *per se*. However, it is certain that since the adoption of antibiotic therapy to combat infectious diseases in the mid-twentieth century, the pace of this evolutionary race has accelerated tremendously. The most relevant player in this game is natural selection, which results from the advantage gained by a mutant organism that can overcome more efficiently the deleterious effects of antibiotics, and also by antibiotic-producers, which are able to find a new way of evading these defenses. In fact, this important role of natural selection has been demonstrated at different scales, from the observed changes in the prevalence of antibiotic resistance genes to the molecular level, by identification of those amino acid residues that are the main targets of selection (Novais et al., 2010).

Notwithstanding the relevance of natural selection, other evolutionary mechanisms are also important in the evolution of antibiotic resistance. Obviously, the most important chance involved in evolutionary success is related to horizontal gene transfer and the integration of antibiotic resistance gene in widespread plasmids and/or clones, but this aspect is not considered in this review. Other chance events such as mutations may play an important role in the evolutionary trajectories followed by antibiotic resistance strains. This has been experimentally shown to occur in the evolution of resistance to β-lactams on at least two occasions. Novais et al. (2010) showed that depending on the first mutation to appear in a few critical positions, resistance to cefotaxime and ceftazidime might follow-up to three alternative routes in CTX-M-type β-lactamases. A similar result was obtained after the *in vitro* evolution of TEM-1 β-lactamase (Salverda et al., 2011).

Apart from contingency, other stochastic processes may also be important for the evolution of antibiotic resistance. In their analysis of CTX-M experimental evolution, Novais et al. (2010) found that Darwinian selection alone could not explain the evolution of the CTX-M variants with the highest resistance to cefotaxime and ceftazidime because the fitness gradient along the different alternative routes always involved at least some steps of reduced fitness. The alternative possibility discussed by Novais et al. implies the action of genetic drift and selection in heterogeneous environments to cross these fitness valleys. In consequence, the action of natural selection does not guarantee, on its own, the evolution of the genotype with the highest fitness. Therefore, it would be of interest to extend these analyses to other antibiotic resistance genes and to evaluate the prevalence and relevance of natural selection and other evolutionary mechanisms in their recent and ancient evolution.

Table 2 | Examples of resistance mechanisms in clinical isolates that evolved from natural functions in environmental bacteria.

Antimicrobial group	Mechanisms	Related natural protein	Natural reservoirs
Aminoglycosides	AcetylationPhosphorylation	Histone-acetylasesProtein kinases	*Streptomyces*
Tetracyclines	Efflux (mar)	Major facilitator superfamily EF-Tu, EF-G	*Streptomyces*
Chloramphenicol	AcetylationEfflux (mar)	AcetylasesMajor facilitatorsuperfamily EF-Tu, EF-G	*Streptomyces*
Macrolides	Target mutation	50S ribosomal subunit	*Streptomyces*
β-lactams (methicillin)	PBP2a	Homologous PBP2a	*Staphylococcus sciuri*
β-lactams (carbapenems)	OXA-48 inactivating enzyme	Proteins participating in peptidoglycan synthesis	*Shewanella xiamenensis*
	OXA-23 inactivating enzyme	Proteins participating in peptidoglycan synthesis	*Acinetobacter radioresistens*
Fluoroquinolones	Topoisomerase protection	Qnr-like protein	*Shewanella algae*

β-Lactams are the most widely used antibiotics in clinical practice over the world. Strong selective pressures upon them have resulted in a continuous increase of resistant isolates (Pitout and Laupland, 2008). In addition, they have also driven the diversification of known β-lactamases genes (Bush and Jacoby, 2010), in order to increase the spectrum of action, and facilitated the acquisition of new mechanisms from the environmental resistome, such as CTX-M β-lactamases (Poirel et al., 2002). Since the 1990s, the identification of new β-lactamase variants has increased dramatically (Davies and Davies, 2010), especially as a consequence of new class A and D β-lactamase variants (Bush and Jacoby, 2010). Moreover, the description of new β-lactamases with carbapenemase activity such as NDM and KPC (*Klebsiella* producing carbapenemase; Bonomo, 2011; Cornaglia et al., 2011), outbreaks of known β-lactamases such as OXA-48 (Dimou et al., 2012) and the spread of *ampC* in plasmids (D'Andrea et al., 2011) have contributed to the current state of alarm (Bush, 2010). We have analyzed, for the better understanding of the factors and processes underlying the recent spread of β-lactamases, the evolutionary dynamics of three β-lactamase families, i.e., ESBL-OXA, CYM, and IMP, representing enzymes belonging to classes D, C, and B, respectively. We decided to exclude the most prevalent (Class A), such as TEM and CTX-M, because they are well characterized (Salverda et al., 2010; Cantón et al., 2012) and their extent diversity is the consequence of recent evolution in response to the clinical use of β-lactam antibiotics (Weinreich et al., 2006; Novais et al., 2010). We have also excluded other worrying cases for public health, such as NDM and KPC, because the diversity of their known variants is still very low for a phylogenetic analysis.

It is already well understood that the OXA family is very diverse (Sanschagrin et al., 1995) and old (Barlow and Hall, 2002b). Only one phylogenetic analysis has been published using 35 OXA β-lactamases (Barlow and Hall, 2002b). The main conclusions of Barlow's work were: first, four branches were identified (OXA-1/OXA-9; OXA-2; OXA-5; OXA-23); second, of the three mobilization events from a chromosomal site to plasmids detected two were ancestral events (OXA-1); and third, three branches (OXA-1, OXA-2, and OXA-10) were under significant positive selection at the beginning of the diversification process. A total of 192 ESBL-OXA sequences downloaded from GenBank were used in our new approach. In order to facilitate the interpretation of the resulting maximum-likelihood (ML) phylogenetic tree, highly supported (BS > 95%) clades of closely related sequences have been grouped (**Figure 2**). Each group has been denoted by a representative OXA variant present therein and the result essentially matches the nomenclature of similar groups in previous works (Couture et al., 1992; Poirel et al., 2010). Five major branches can be distinguished now. The new branch identified corresponds to OXA-61/63. The OXA-1/OXA-9 branch shows two different dynamics. The OXA-9 clade differs from other previous groups in its ancient origin, with long internal branches indicating long periods of evolution after divergence from the corresponding ancestors. On the contrary, the OXA-1 clade includes sequences from several different species such as *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and others. They are very similar and suggest a very recent spread from a common ancestor. The OXA-51/OXA-23 branch includes 120 sequences, all but one from *Acinetobacter*. From these results, it is likely that this group of sequences originated in *Acinetobacter* and has remained distributed mostly within this genus. The only exception corresponds to a plasmid-borne representative isolated in *Klebsiella*, where it probably arrived from an *Acinetobacter* ancestor. The OXA-51 clade, the largest subgroup, is a very homogeneous group of sequences, with an average nucleotide distance of 0.018 substitutions/site (s/s) and a maximum within-group distance of 0.036 s/s. These results indicate that this group has diversified recently. Similarly, recent diversification events were suggested in the OXA-10 (the second largest group with 18 sequences) and OXA-2 clades. The average pairwise divergences were 0.022 s/s and 0.132 s/s and a maximum value of 0.045 s/s and 0.288 s/s, respectively. The OXA-54 clade, with very recently evolved sequences, was also found to be closely related to the OXA-2 clade.

These results are indicating that only some branches are evolving quickly and they could be under positive selection. Hence, the selective processes might have left an imprint in the form of increased rates of non-synonymous substitutions (dN) compared to those of synonymous substitutions (dS). In consequence, we have tested this hypothesis by comparing the rates of dS and dN substitutions at each codon. Based on the ML tree, estimates of the two substitution rates were obtained using the ancestral states inferred by ML under a Muse–Gaut model (Muse and Gaut, 1994) of codon substitution and the Tamura–Nei model of nucleotide substitution (Tamura and Nei, 1993). Computation of dN/dS was performed with the Hyphy software package (Kosakovsky Pond et al., 2005). Positions with <95% sequence coverage (i.e., with >5% of the sequences presenting a gap) were removed from the analysis. In contrast to the initial expectation, no codon presented a statistically significant higher rate of dN than dS substitutions. The same general results were obtained when the different groups in the OXA phylogenetic tree were analyzed separately in search of positively selected codons as well as in an analysis with the seven ungrouped sequences of this gene (**Figure 1**). This result indicates that neither the ancient divergence nor the recent spread of some groups of OXA alleles have been driven primarily by selection acting at this (codon) level.

Plasmid-encoded *ampC* genes have been known since 1989 when CMY-1 was described in *K. pneumoniae* isolates (Bauernfeind et al., 1989), while the most common plasmid-mediated AmpC β-lactamase worldwide is CMY-2 (Jacoby, 2009). Generally, CMY-2 enzymes are responsible for outbreaks across European countries (D'Andrea et al., 2011). CMY-1 and CMY-2 have different phylogenetic origins (**Figure 3A**); whereas CMY-1 and related variants are close to chromosomally determined AmpC enzymes in Aeromonadaceae, CMY-2 and evolved variants are related to AmpC from *Citrobacter freundii* (Barlow and Hall, 2002a). A total of 32 CMY alleles with known date and country of first isolation were included in this analysis. These variants cluster into two well-defined and distant groups in the reconstructed ML tree using the Tamura-3P + gamma model (**Figure 3**), denoted CMY-1 and CMY-2. The CMY-2 group is larger and more diverse. A large number of sequences ($n = 22$) from the five continents are very similar and have diversified very recently, while the five remaining sequences in the CMY-2 group have a more ancient origin. This larger group is dominated by isolates from *E. coli*, but a recent

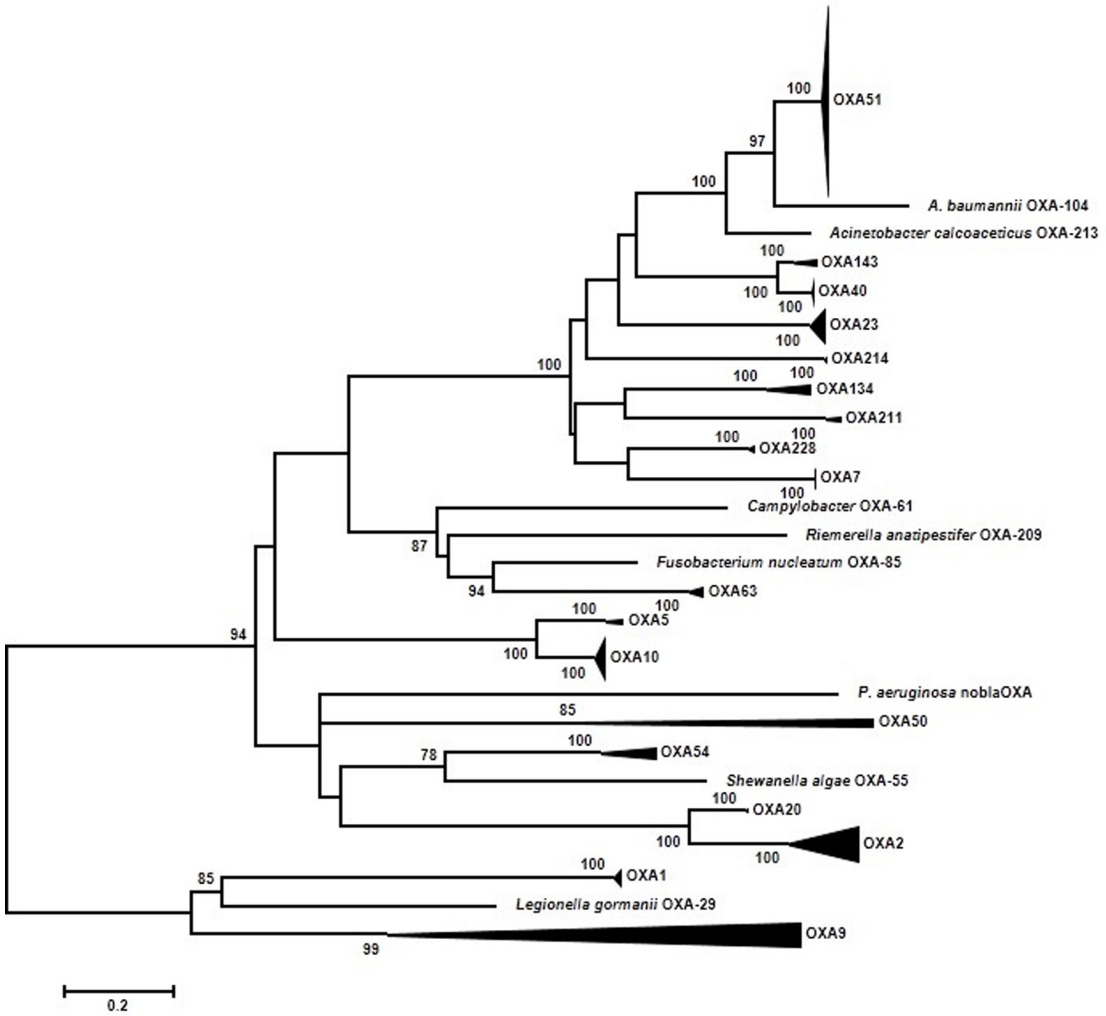

FIGURE 2 | Summarized representation of the maximum-likelihood tree of OXA genes obtained with the K2P + GI model of evolution. Multiple nucleotide sequence alignments were obtained using the corresponding amino acid sequences with Muscle (Edgar, 2004a,b). Maximum-likelihood trees were obtained using the most appropriate model of evolution resulting from the comparison of Bayesian Information Criterion (BIC) values for 24 alternative models including gamma-distributed heterogeneous rates of evolution among and invariant sites. Support of the inferred clusters was evaluated with 1000 bootstrap replicates; BS values >70% are indicated. All these methods were used as implemented in MEGA 5 (Tamura et al., 2011). The scale bar corresponds to substitutions/site. The role of natural selection was investigated at the codon level by analyzing the difference between dN–dS substitution rates at different positions. Based on the corresponding maximum likelihood trees using a Muse–Gaut model (Muse and Gaut, 1994) of codon substitution and the Tamura–Nei model of nucleotide substitution (Tamura and Nei, 1993). Computation of dN/dS was performed with the Hyphy software package (Kosakovsky Pond et al., 2005). Additionally, two tests of neutrality were applied to the three data sets: the Fisher's exact test of neutrality and a test based on bootstrapping (1000 replicates) of dN–dS values from which a variance of the corresponding statistic is derived in order to test the null hypothesis of neutrality (dN = dS). These tests were performed using MEGA 5 (Tamura et al., 2011).

colonization and spread into *Proteus mirabilis* can be observed in sequences isolated from European countries (D'Andrea et al., 2011) with a possible origin in North Africa (CMY-4 from Tunisia). The CMY-1 group included only six sequences from Asian–Pacific countries, which are distributed evenly in two subgroups of very similar sequences.

The CMY alignment resulted in 376 codons available for selection analysis. None of them presented significant deviations from the expected dN−dS value under neutrality, although 123 codons had positive values for this difference. These analyses corresponded to the whole data set of CMY alleles considered but, as shown above, there are two main, anciently-diverged groups that have been analyzed separately in search of positive selection. The phylogeny-based analysis of CMY-1 variants failed to identify any codon with a significant positive difference between dN and dS, with only seven codons yielding positive values of this parameter none of which reached statistical significance. Similar results were obtained for the CMY-2 group, with average dN–dS values of −0.362 (range: −10.484 to 1.000, minimum $P = 0.444$), and the two pairwise comparison-based tests failing to reveal evidence for positive selection. Barlow and Hall (2002a) obtained similar results using a lower number of variants. Although a diversification

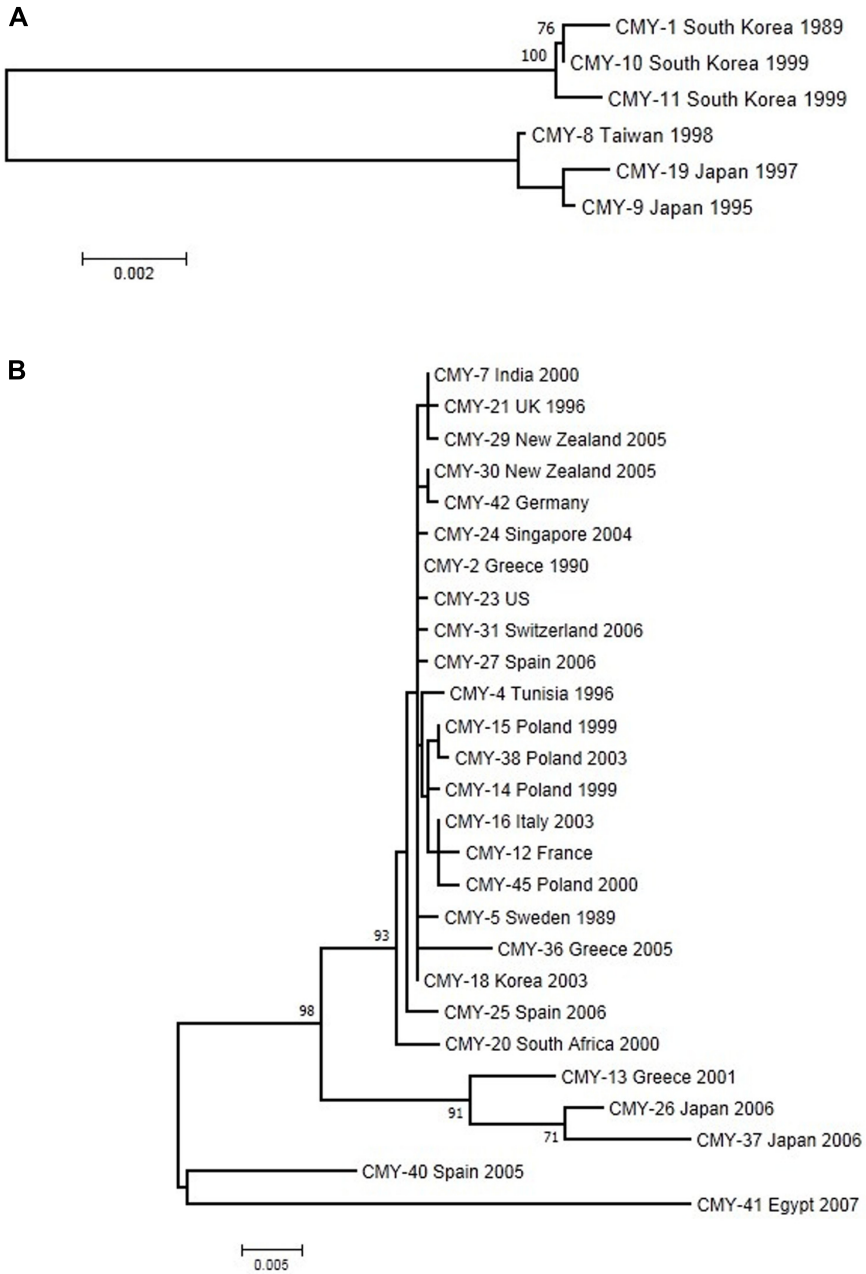

FIGURE 3 | Maximum-likelihood tree of CMY genes obtained with the Tamura-3P + G model of evolution. (A) The upper tree corresponds to CMY-1 group. **(B)** The lower one to the CMY-2 group of sequences. Multiple sequence alignments were obtained using the corresponding amino acid sequences with Muscle (Edgar, 2004a,b). Maximum-likelihood trees were obtained using the most appropriate model of evolution resulting from the comparison of Bayesian Information Criterion (BIC) values for 24 alternative models including gamma-distributed heterogeneous rates of evolution among and invariant sites. Support of the inferred clusters was evaluated with 1000 bootstrap replicates, BS values >70% are indicated. All these methods were used as implemented in MEGA 5 (Tamura et al., 2011). The scale bar corresponds to substitutions/site. Detection of positive selection and neutrality tests were performed as described in **Figure 2**.

process has occurred in this group, especially in CMY-2, the newly arisen variants were not subject to positive selection, in contrast to TEM and CTX-M enzymes. The hydrolytic activities conferred by chromosomal *ampC* recovered from the pre-antibiotic era are essentially the same as plasmid-mediated *ampC* alleles, probably because they are all resistant to many β-lactam antibiotics. Therefore, the plasmid-mediated AmpC enzymes are not evolving phenotypically because it is not necessary (Barlow and Hall, 2002a). However, the impact of single-amino acid substitutions on the evolution of CMY proteins can be observed

in particular cases. For instance, the G214E change in CMY-2 increases the catalytic efficiency against cefotaxime (Endimiani et al., 2010), suggesting that although the selective pressure occurs it might be weak.

β-Lactamases with capacity for hydrolyzing carbapenems are very versatile in their origins and nucleotide sequence. Currently, almost 30 families of carbapenemases belonging to classes A, D, and B are known (Widmann et al., 2012), but new families are being described continuously (Pollini et al., 2012). The spread of metalo-β-lactamases (MBL), classified as class B, presents a major challenge both for treatment of individual patients and for policies of infection control (Cornaglia et al., 2011), because they confer resistance to almost all β-lactams, except aztreonam. There are at least nine different types of MBL, but probably IMP and VIM are the most prevalent (Fukigai et al., 2007; Van der Bij et al., 2011) and diversified[1]. The dendrogram of VIM enzymes suggests two recent events of diversification involving VIM-1 and VIM-2 (Cornaglia et al., 2011).

We have analyzed 21 IMP alleles with year and country of first isolation. The ML tree was constructed using the Tamura-3P model. According to this phylogeny (**Figure 4**), several closely related groups of likely recent origin and many other more anciently diverged sequences can be observed. The two recently diverged groups include mostly sequences isolated from East Asian countries. A common feature of these recently spread groups is that they include most alleles isolated in species not belonging to *P. aeruginosa* and *A. baumannii*. The first IMP allele, IMP-1, was isolated in Japan and it is included in a group with three additional alleles from the same country. Another group related to this one includes two sequences isolated in Hong Kong and Singapore, respectively. The second recently spread group includes two sequences from Taiwan and one from Italy and France. In both groups, the V67F change was selected on three occasions suggesting that this change could be under positive selection. However, no mutation presented a statistically significant, positive deviation in the dN–dS parameters indicative of positive selection acting in the IMP phylogeny. Pairwise comparisons also failed to reveal positive selection as a driving force in the evolution of these alleles. The V67F change is highly variable, non-essential for the protein function but it could have a role in antibiotic recognition (Widmann et al., 2012). Moreover, in the IMP-1 clade, the mutation G235S contributes in increasing the hydrolytic activity against meropenem (Liu et al., 2012), suggesting a process of selection.

Despite their many differences at the genetic and ecological levels, the three families of β-lactamases present several remarkable similarities. The three gene families have several anciently diverged variants along with others that have spread very recently. Most of these recently diverged groups include very similar sequences with only a few nucleotide substitutions and usually only one or two amino acid replacements. This distribution probably results from a biased screening of variants – only those with at least one amino acid difference are reported and deposited in the corresponding databases, which may lead to the false impression that positive selection is a major factor driving the evolution of these genes. In fact, our analyses in search of positive selection in these gene families failed to find evidence for it after using both phylogeny- and pairwise-based analysis of dN–dS differences in codons or the whole gene. In general, most isolates in recently spread OXA- and IMP-groups are derived from *A. baumannii* and *P. aeruginosa*. Over 210 and 120 β-lactamases have been identified in *A. baumannii* and *P. aeruginosa*, respectively, suggesting that these species are crucial reservoirs of β-lactam resistance determinants (Zhao and Hu, 2010, 2012). Although they are ubiquitous in nature also they are frequently isolated in nosocomial and chronic infections, exposed to a variety of antibiotic regimens, giving rise to selection and spread of mechanisms of resistance. In this scenario these results suggest that these species are an excellent shuttle of resistance genes between the environment and pathogenic strains.

UNDERSTANDING THE ADAPTIVE POSSIBILITIES AND THEIR CONSTRICTIONS TO PREDICT β-LACTAMASE EVOLUTION

The ability of protein to evolve is dependent on tolerance to change. TEM β-lactamases can be extremely tolerant to amino acid substitutions both in the number of affected positions (>200 positions can change; Salverda et al., 2010) as in number of amino acids in a particular position (one-third of the positions in TEM can tolerate more than five different amino acids; Deng et al., 2012). The combination of potential mutations could increase the theoretical diversity up to astronomically large numbers. However, only 82 polymorphic positions have been described among the 204 TEM mutants described so far and, strikingly, 85% of the substitutions are located in only 17 positions.[1] Moreover, the most polymorphic positions show only 3–4 amino acid changes. So, why does not the sequence plasticity observed in site-directed mutagenesis experiments translate easily in huge diversification of evolved proteins in nature? (Taverna and Goldstein, 2002).

Many researchers have studied the possible genetic constraints involved in the discrepancy between the potential and the actually observed biological plasticity of β-lactamases (Camps et al., 2007). Mutagenesis studies on TEM-1 did not yield TEM variants more efficient than TEM-1 in hydrolyzing natural β-lactams such as ampicillin and cephalosporin C, suggesting that during thousands of years of evolution TEM-1 has become the most efficient enzyme in conferring the highest MIC values to natural β-lactams (Deng et al., 2012). The kinetic effect of this *evolved enzyme* is the result of a delicate network of hydrogen bonds contributing to the enzymatic stabilization. Therefore, it is easy to imagine that any mutation could alter this balance and consequently decrease its efficiency. Internal positions (close to the active center) are less tolerant of substitutions than external ones because, presumably, they have more interactions and the equilibrium is altered easily (Deng et al., 2012). Therefore, the majority of mutations in internal positions are generally deleterious but they might also facilitate the acquisition of new functions (Tokuriki et al., 2008). For instance, mutations such as R164H in TEM-1 (TEM-29), D179N in SHV-1 (SHV-8), or P167T in CTX-M-1 (CTX-M-58) increase the hydrolytic activity against third generation β-lactam antibiotics (*new* function), but decrease the specific activity of the existing function as a consequence of a loss of stability (Poirel et al., 2001; Wang et al., 2002). The protein instability

[1] http://www.lahey.com

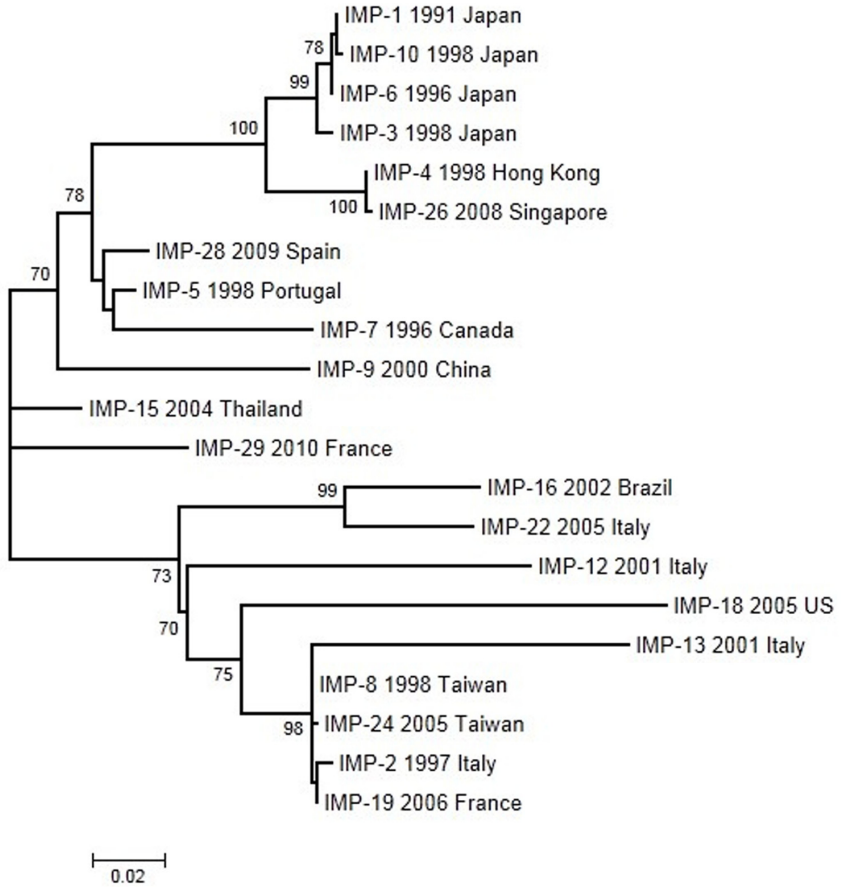

FIGURE 4 | Maximum-likelihood tree of IMP genes obtained with the Tamura-3P model of evolution. Multiple sequence alignments were obtained using the corresponding amino acid sequences with Muscle (Edgar, 2004a,b). Maximum-likelihood trees were obtained using the most appropriate model of evolution resulting from the comparison of Bayesian Information Criterion (BIC) values for 24 alternative models including gamma-distributed heterogeneous rates of evolution among and invariant sites. Support of the inferred clusters was evaluated with 1000 bootstrap replicates, BS values >70% are indicated. All these methods were used as implemented in MEGA 5 (Tamura et al., 2011). The scale bar corresponds to substitutions/site. Detection of positive selection and neutrality tests were performed as described in **Figure 2**.

observed with some mutations leads to lower levels of correctly-folded protein and, consequently, to a reduction in enzymatic activity (Bloom et al., 2005). Therefore, the stability/instability level of a mutant protein must be proportional to its fitness. Almost 40% of TEM-1 artificial mutations cause a partial or complete reduction in the level of folded protein (Tokuriki and Tawfik, 2009). Hence as mutations accumulate, the likelihood of declines in protein fitness increases exponentially (Soskine and Tawfik, 2010).

According to the theoretical estimations, those proteins carrying, five mutations on average, with respect to the wild-type variant, will reduce their fitness in > 80% (Tokuriki and Tawfik, 2009) and the accumulation of >10 mutations per gene would result in non-functionalization of 99% of the mutated genes (Soskine and Tawfik, 2010). However, several TEM-1 variants, such as TEM-121, TEM-162, or TEM-194, carrying five or more mutations have been described in nature. The increased tolerance to changes observed in these enzymes requires the presence of mutations capable of compensating the loss of stability due to previous selection of mutations conferring *new* functions (Kather et al., 2008). In other words, the evolution toward *new* functions must be facilitated by mutations that act as compensatory mutations of protein stability defects (Brown et al., 2010).

The best studied example of compensatory mutation is M182T in TEM enzymes. This mutation has no effect on the catalytic activity of TEM-1 (Huang and Palzkill, 1997), however the thermodynamic stability of TEM-1 is increased by 2.67 kcal/mol in the presence of this mutation (Wang et al., 2002). Therefore, the selection of M182T will be favored when previous mutations that increase the activity against new β-lactams have reduced protein stability (Camps et al., 2007). For instance, TEM-15 has substitutions E104K:G238S which contribute synergistically to increase the hydrolytic activity toward extended-spectrum cephalosporins. These mutations rearrange the active site and, consequently, destabilize the enzyme ($\Delta\Delta G = 2.24$ kcal/mol), whereas the triple mutant, TEM-52, contains the additional substitution M182T compensating the loss associated with the E104K:G238S

substitutions ($\Delta\Delta G = -1.76$ kcal/mol), increasing the activity against extended-spectrum β-lactams (Wang et al., 2002). Progressively, other compensatory mutations have been described in TEM enzymes (Brown et al., 2010).

The presence of compensatory mutations is a global phenomenon, also described in other β-lactamases, such as mutations A77V in CTX-M (Novais et al., 2008), N70S in metallo-β-lactamase BcII (Tomatis et al., 2008), or L169I in OXA and ROB-β-lactamases (Poirel et al., 2002; Galán et al., 2003). From an evolutionary perspective, these point mutations are essential because the *extra* stability promotes the evolvability of the protein (Bloom et al., 2006), but potentially they can also have negative effects if the excess stability hinders protein turnover (Brown et al., 2010). Therefore, an equilibrium between evolvability and robustness is essential for the plasticity of proteins, that is, between the necessity to adapt to new environments and the ability to maintain a phenotype in the presence of genotypic variations.

The network model establishes strong associations between particular mutations. For instance, mutation M182T is more frequently associated with mutations related to ESBL phenotypes (Huang and Palzkill, 1997), whereas N276D is associated with IRT phenotypes (Abriata et al., 2012), suggesting co-evolutionary processes (Guthrie et al., 2011). In other cases, mutation L210P is co-selected with R244A (Marciano et al., 2008), whereas mutation E240K is associated with R164H, suggesting different networks of compensatory mutations depending on the initial mutation responsible for the ESBL phenotype. These epistatic constrictions required for maintaining stability while activity is increased drastically reduce the number of possible mutational pathways (Weinreich et al., 2006; Novais et al., 2010). Therefore, initial mutations drive evolutionary pathways of protein evolution (Salverda et al., 2011). The genetic reconstruction based on a combinatorial strategy of five mutations in TEM-1, which were chosen for their large joint phenotypic effect, revealed that only 18 of 120 evolutionary trajectories were accessible through Darwinian selection (Weinreich et al., 2006). Our group, using the CTX-M enzyme as model, obtained similar results but concluded that only the simultaneous presence of cefotaxime and ceftazidime in the environment permitted reaching the highest level of resistance (Novais et al., 2010). These studies of experimental evolution on β-lactamases show that only a small fraction of all possible mutational trajectories are accessible to evolution.

These lines of evidence suggest that many combinations of mutations must be antagonistic. Pleiotropic antagonism has been used to describe the incompatibility between ESBL and inhibitor-resistant β-lactamases phenotypes (Cantón et al., 2008; Ripoll et al., 2011). However, in this case pleiotropic antagonism is defined as the incompatibility between mutations conferring the same phenotype. For instance, mutations D240G and P167S increase the hydrolytic activity of CTX-M enzymes to ceftazidime. However, the double mutant confers lower MIC values than both single mutants in every background (Novais et al., 2010). Similar results were observed between mutations R164S and G238S in TEM, which show a strong negative interaction (Salverda et al., 2011). Laboratory-directed protein evolution experiments revealed that P167S and G238S mutations were more frequently selected (Barlow and Hall, 2002c; Novais et al., 2008) which are known to cause the greatest increase in CAZ and CTX resistance among all known single CTX-M and TEM mutations. However, there are more clinical isolates carrying the D240G and R164S mutations in CTX-M and TEM, respectively. This observation is contradictory to the Darwinian paradigm of evolution, as genotypes with higher fitness must be favored by selection ("survival of the fitness").

At high mutation rates or weak bottlenecks, selection favors genotypes with larger networks of interactions, which will be more tolerant (robust) to the impact of deleterious mutations in spite of lower fitness. This phenomenon is known as "survival of the flattest" (Codoñer et al., 2006; Archetti, 2009). Depending on the environmental conditions, selection may favor an organism that replicates faster ("survival of the fitness") or is more robust ("survival of the flattest"), but not both at the same time (Codoñer et al., 2006). The high connectedness of robust genotypes will be a guarantee of innovation without drastically losing functionality in the long time scale (Ferrada and Wagner, 2008), whereas a high evolvability is a guarantee for survival to strong bottlenecks in a short time but at the cost of losing many interactions and *old* functions. In general, β-lactamases carrying D240G or R164S have accumulated more mutations and the *old* functions are less affected than in β-lactamases harboring P167S or G238S mutations in the first step. Therefore, D240G or R164S mutations in CTX-M and TEM enzymes will condition the "survival of the flattest" model; whereas P167S or G238S mutations will contribute to "survival of the fittest." These results suggest that the first mutations not only determine the co-selection of specific compensatory mutations but also condition the selection of certain evolutionary strategies. Nevertheless, the first mutations to be selected will also depend on the frequency and intensity of changes in the environment.

Antibiotic resistance is a public health problem and for many microbiologists and evolutionary researchers a goal to address the resistance problem has been to predict the selection of antibiotic resistance to new antibiotic (Martínez et al., 2007). In this situation, which types of studies are necessary to analyze in more depth the development of resistance in human bacterial pathogens? For many years, studies based on serial-passages experiments have been used as the best model to predict the selection of mutations involved in extending the spectrum of enzymatic activity (Martínez et al., 2011). However, this approach generally detected single mutants and, as a consequence of the strong bottlenecks involved in this experimental design, in most cases only those fitness variants corresponding to the "survival of the fitness" concept were recovered (Novais et al., 2008; Ripoll et al., 2011). This simple vision does not predict the selection of complex mutants based on the "survival of the flattest" concept. Then, the evolvability of known antibiotic resistance genes was explored by DNA shuffling and error-prone PCR (Orencia et al., 2001). The combination of both approaches in fluctuating environments could be more realistic, because in these cases mutations representing the "survival of the flattest," such as R164S, are easily selected (Blázquez et al., 2000; Barlow and Hall, 2002c). Two new approaches are contributing to improve our predictive capacity. One of them, defining the driving forces the selection in a given combination of time and environment and understanding the possible evolutionary

trajectories in the diversification process (Weinreich et al., 2006; Novais et al., 2010). Secondly, the implementation of bioinformatics programs such as FoldX (Deng et al., 2012), determining the ΔΔG of a new mutant and consequently its stability and fitness. The combinatorial approaches between bioinformatics and *in vitro* procedures will give rise to a more complete vision on the complex process of antibiotic resistance evolution.

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Evolutionary consequences of antibiotic use for the resistome, mobilome, and microbial pangenome

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The widespread use and abuse of antibiotic therapy has evolutionary and ecological consequences, some of which are only just beginning to be examined. One well known consequence is the fixation of mutations and lateral gene transfer (LGT) events that confer antibiotic resistance. Sequential selection events, driven by different classes of antibiotics, have resulted in the assembly of diverse resistance determinants and mobile DNAs into novel genetic elements of ever-growing complexity and flexibility. These novel plasmids, integrons, and genomic islands have now become fixed at high frequency in diverse cell lineages by human antibiotic use. Consequently they can be regarded as xenogenetic pollutants, analogous to xenobiotic compounds, but with the critical distinction that they replicate rather than degrade when released to pollute natural environments. Antibiotics themselves must also be regarded as pollutants, since human production overwhelms natural synthesis, and a major proportion of ingested antibiotic is excreted unchanged into waste streams. Such antibiotic pollutants have non-target effects, raising the general rates of mutation, recombination, and LGT in all the microbiome, and simultaneously providing the selective force to fix such changes. This has the consequence of recruiting more genes into the resistome and mobilome, and of increasing the overlap between these two components of microbial genomes. Thus the human use and environmental release of antibiotics is having second order effects on the microbial world, because these small molecules act as drivers of bacterial evolution. Continued pollution with both xenogenetic elements and the selective agents that fix such elements in populations has potentially adverse consequences for human welfare.

Keywords: metagenomics, evolvability, pollution, pangenome, resistome, parvome, mobilome

INTRODUCTION

The discovery of antibiotics and their use in the treatment of bacterial infections was one of the major scientific achievements of the 20th century. However, over the last 60 years, there has been a spectacular and rapid evolution of antibiotic resistant strains of bacteria. This has culminated in the appearance of pathogens with resistance to a wide range of antibiotics, and a rise of similarly resistant opportunistic organisms (Davies, 2007; Davies and Davies, 2010). Antibiotic resistance is a critical problem for humans and their domestic animals, and is recognized as such by workers in government, clinical practice, research, and industry (Bush et al., 2011).

The focus of research and thinking about this problem has mainly been from an anthropogenic viewpoint. However, at its heart, antibiotic resistance is an environmental and evolutionary problem. Humans are now the greatest evolutionary force on the planet (Palumbi, 2001), and it would help us understand and manage the resistance problem to investigate resistance from a broader perspective, most notably in terms of the interplay between ecology, evolutionary dynamics, and natural selection. A number of authors are now thinking about the ecology and natural history of antibiotics and resistance genes. This approach will inform clinical and veterinary practices and will improve our understanding of evolutionary processes (Aminov and Mackie, 2007; Baquero et al., 2009; Fajardo et al., 2009; Stokes and Gillings, 2011).

There is evidence that the antibiotic revolution may have second order effects, and is influencing the evolution of the entire microbial biosphere (Martinez, 2008; Baquero, 2009; Couce and Blázquez, 2009; Gillings and Stokes, 2012). This paper attempts to place antibiotics, resistance genes, their vectors, and their hosts into an evolutionary perspective. By understanding the evolutionary history of these genes and molecules, we place ourselves in a better position to predict their future (Martinez et al., 2007; Courvalin, 2008; Conway Morris, 2010).

THE NATURAL HISTORY OF ANTIBIOTICS

The term "antibiotic" reflects our anthropocentric viewpoint. Antibiotics are not a discrete class of molecules, but rather, encompass a broad range of structural and molecular families, united by their ability to inhibit microbial growth at high concentrations. The original use of the word "antibiotic" was a generic term that simply reflected the outcome of a laboratory test (Waksman, 1973; Davies and Davies, 2010). In modern terms, an antibiotic has broadly come to mean any synthetic or naturally occurring low molecular weight molecule that inhibits bacterial growth.

It is clear that there are millions of low molecular weight compounds in natural environments, and that in high enough

concentrations some of these could exhibit an antibiotic effect. However, it is unlikely that such compounds ever reach inhibitory concentrations in nature, and there is a growing realization that their primary role is not necessarily in cross-species warfare, but may largely lie in cell–cell communication (Linares et al., 2006). This change in viewpoint began with the discovery of bacterial quorum sensing, a system that allows communication between cells of the same or different species via signaling molecules called auto-inducers. Quorum sensing is found in diverse organisms, and the scale and extent of signaling systems in general has led to the revelation that the microbial world is in a state of constant and complex communication (Lyon and Muir, 2003; Henke and Bassler, 2004; Stevens et al., 2012). Consequently, many of the small molecules produced by bacteria may be involved in additional forms of communication between cells. The compounds we know as "antibiotics" are a minor subset of this world of small molecules.

The growth-inhibiting concentrations of antibiotics used in clinical practice are unlikely to ever be reached via natural synthesis, and consequently, antibiotics probably have significant effects at sub-inhibitory concentrations, without affecting bacterial growth rates. It has now been shown that sub-inhibitory concentrations of various antibiotic classes have effects on gene transcription. Up regulation and down regulation of diverse genes has been demonstrated, with some estimates suggesting that as many as 5% of gene promoters might be affected (Goh et al., 2002; Linares et al., 2006). Some antibiotic classes modulate expression of particular genes (Tsui et al., 2004). The types of functions affected are diverse, including genes for protein synthesis, carbohydrate metabolism, transport/binding proteins and genes of as yet unknown function (Goh et al., 2002; Lin et al., 2005).

The cascade of gene expression induced by sub-inhibitory concentrations of antibiotics leads to phenotypes of adaptive significance. These include flagella biosynthesis and the ability to colonize biotic and abiotic surfaces (Seshasayee et al., 2006). As might be expected, some of these phenotypes have direct relevance for species–species interactions and responses to host cells, such as virulence, biofilm formation, and motility (Goh et al., 2002; Hoffman et al., 2005; Linares et al., 2006; Marr et al., 2007; Shank and Kolter, 2009). Collectively, these observations strongly suggest that the antibiotics used in medical treatment are a small subset of the diverse secondary metabolites used in the microbial world for signaling and other cell–cell interactions. Their clinical use is based on a serendipitous ability to inhibit growth at unnaturally high concentrations. There is a growing realization that the discovery, clinical use, and evolution of antibiotics must be considered against the background of their roles in natural environments (Davies et al., 2006; Yim et al., 2007; Fajardo and Martínez, 2008; Ryan and Dow, 2008).

A GENERIC MODEL OF SMALL MOLECULE SIGNALING

Thinking about antibiotics as a subset of signaling molecules allows us to construct simple conceptual models that might establish some general principles about antibiotics and antibiotic resistance (**Figure 1**). A signaling molecule must be made by a metabolic pathway, whose enzymes are encoded by genes (*A, B,* and *C* in **Figure 1**). Any intermediate small molecule in this pathway can be exported as a signaling molecule, most probably using an efflux pump. Small molecules then diffuse into environmental space, where they can bind to receptors on the cell surface of the producing species (intra-species signaling), or to cell surface receptors of different species (inter-species signaling). Alternatively, small molecules can be imported into cells via a membrane transport protein, and then bind to a target within the cell (**Figure 1**). Binding of signaling molecules can influence transcription or biochemical pathways, and thus affect the phenotypic attributes of the receptor cell.

This conceptual model (**Figure 1**) allows us to consider antibiotics and resistance from a different and broader perspective. Antibiotics can be thought of as a subset of small biosynthetic molecules, albeit those which happen to have the property of inhibiting bacterial growth at high concentrations. Given the fact that bacteria have been evolving and interacting for some 3.8 billion years, it should not be surprising that the genes encoding biosynthetic and catabolic pathways for small molecules have an ancient evolutionary history. Pathways for synthesis of erythromycin and streptomycin date to more than 600 million years ago (Baltz, 2005), and it has been estimated that the serine β-lactamases date back 2 billion years (Hall and Barlow, 2004). Under this view "antibiotics" predate humans by some billions of years.

Note that the use of an intermediate in the biosynthetic pathway as a signaling molecule (**Figure 1**, triangle) means that

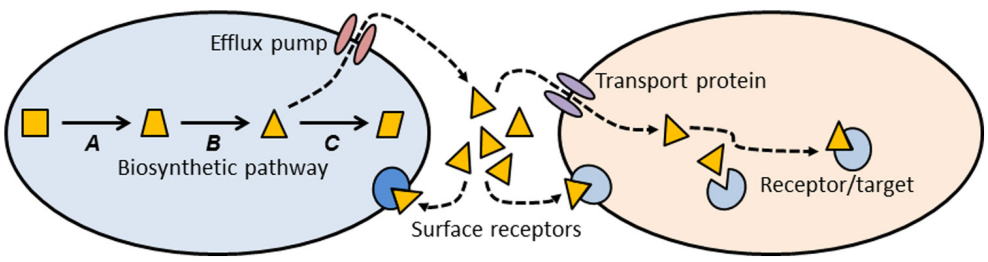

FIGURE 1 | Conceptual schematic illustrating the production, export, and target sites of a small molecule biosynthetic cluster. The metabolic pathway for synthesis of a small molecule is encoded by genes *A, B,* and *C*. One of the intermediates (triangle) is exported from the cell via an efflux pump, where it can then bind to cell surface receptors, or enter a second cell via a membrane transport protein. Inside the second cell there may be binding sites on additional target molecules. Most of the molecules we know as antibiotics may be a subset of this more general class of signal-receptor systems.

the producing cell already encodes an enzyme capable of modifying the signal molecule. Under other circumstances, such a gene might confer resistance to high concentrations of the agent. Many biosynthetic gene clusters that make "antibiotics" are also known contain genes that confer "resistance" to those same antibiotics (Pootoolal et al., 2002; Huang et al., 2005). Given that gene clusters for secreted molecules are frequently subject to lateral gene transfer (LGT; Fischbach et al., 2008; Nogueira et al., 2009), it should not be surprising that there is clear evidence for lateral transfer of antibiotic resistance genes from environmental bacteria into pathogens (Gillings et al., 2008a,b; Forsberg et al., 2012).

Small molecules can exit the producing cell via a wide diversity of membrane bound efflux pumps (**Figure 1**; Paulsen et al., 1996; Desvaux et al., 2004; Poole, 2005; Bay and Turner, 2009), each of which, in turn, may be able to export diverse molecular species (Zgurskaya and Nikaido, 2000; Kuete et al., 2011). Such pumps have evolved as mechanisms to export natural substances produced internally, or those that are produced by other cells (Martinez et al., 2009b). Efflux pumps may also be essential for colonizing and persisting in eukaryotic hosts (Piddock, 2006). It is thus clear that antibiotic resistance mediated by efflux pumps is a side-effect of a more general molecular export system.

Genes for efflux pumps are commonly found in bacteria that produce antibiotics (Petković et al., 2006), and these genes are often found embedded within biosynthetic gene clusters for antibiotics, thus conferring efflux ability (and "resistance") on the producing organism (Méndez and Salas, 2001). Again, the "resistance" gene is preexistent, and located within a gene cluster characterized by LGT (Nogueira et al., 2009).

Once exported from the cell, a signaling molecule is free to bind with receptors on the surface of neighboring cells (**Figure 1**). Point mutations in the genes for these receptors might alter the binding site and thus generate a "resistance" phenotype. Alternatively, the signaling molecule may be taken up by a membrane transport protein and imported into a new cell. A surprising diversity of bacteria make use of such transport proteins to import small molecules such as antibiotics, which they can then use as a sole carbon source (Dantas et al., 2008). Lateral transfer of the genes for such catabolic processes would confer resistance on the recipient cell. Resistance could also be generated by mutation to the gene encoding the membrane transport protein, preventing entry of the signaling molecule. Once inside a recipient cell, a signaling molecule may bind to a target site, and thus exert an effect. Mutation of the gene encoding the target site may abolish binding and prevent the effect. In the case of an antibiotic, such a change would be called a resistance mutation.

Although the model I have presented above is simplistic, nevertheless it has good explanatory power for understanding the ecology and evolution of both antibiotics and their corresponding resistance genes. Antibiotics are seen as a subset of a diverse group of small molecules whose primary function is in cell–cell interactions. Genes for synthesizing, transporting, and catabolizing these small molecules can be co-opted as resistance genes when cells are exposed to unnaturally high concentrations of these compounds. The high frequency of lateral exchange of biosynthetic gene clusters for small molecules (together with their associated efflux pumps and catabolic functions) explains the penetration of efflux pumps and degradative enzymes into species unrelated to the original "antibiotic" producer.

ANTIBIOTICS, RESISTANCE GENES, AND THE GLOBAL MICROBIOME

In thinking about the ecology and evolution of antibiotics and resistance genes, we can summarize the sub-components upon which selection can act in a series of Venn diagrams (**Figure 2**). The largest category is the **Global Microbiome**, encompassing all the prokaryotic cells in the biosphere. This may contain $4–6 \times 10^{30}$ cells and hold a significant proportion of the carbon, nitrogen, and phosphorus found in living things (Whitman et al., 1998). The phenotypes exhibited by the global microbiome are encoded by the microbial **Pangenome**, that is, the set of genes present in all the genomes of all the prokaryotes in the biosphere (Medini et al., 2005; Tettelin et al., 2008; Lapierre and Gogarten, 2009). Estimates of the composition of the pangenome have been made, based on the rapid accumulation of bacterial genome and metagenome sequences. Some 250 gene families are common to all bacterial genomes (the extended core genome) and about 8,000 gene families are niche-specific genes essential for survival in particular environments (the character genome). The bulk of pangenomic diversity comprises more than 139,000 gene families that occur as accessory genes, these being found dispersed amongst single strains, serovars or species (Lapierre and Gogarten, 2009). The combined coding capacity of the pangenome is expressed as the **Panproteome**, this being the sum of all the proteins encoded and produced by the microbial realm.

A proportion of the pangenome is highly mobile, and able to move comparatively freely between prokaryote species in the process known as LGT. Elements that specialize in moving DNA within and between genomes include plasmids, transposons, integrons, insertion sequences, and integrative conjugative elements. These elements and the genes they carry are collectively known as the **Mobilome** (**Figure 2**; Siefert, 2009; Leplae et al., 2010). In principle, and over evolutionary timescales, any part of the pangenome can be mobilized by LGT. The mobilome helps to generate enormous diversity within prokaryote genomes by recombination between various mobile elements to create a series of complex mosaic structures (Garriss et al., 2009; Toleman and Walsh, 2011; Toussaint and Chandler, 2012).

The **Resistome** is defined as the collection of all genes that could contribute to a phenotype of antibiotic resistance (D'Costa et al., 2006; Wright, 2007, 2010; Forsberg et al., 2012). This is a purely functional definition made from a human and medical point of view, for as we have seen, the resistome encompasses diverse genes whose original functions were not simply for avoiding the effects of antibiotics. Nevertheless, it is a useful concept, because it underscores the point that resistance genes originate from environmental bacteria. A database of antibiotic resistance genes has been developed (Liu and Pop, 2009), but the 20,000 genes already listed are only a small proportion of the resistome, since investigation of soil metagenomes has revealed diverse and divergent resistance genes capable of dealing with multiple classes of antibiotic (Riesenfeld et al., 2004; Allen et al., 2008). Genes encoding

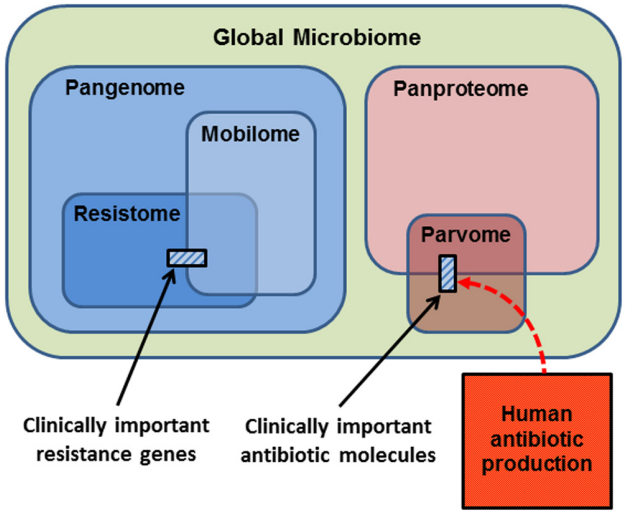

FIGURE 2 | Conceptual representation of the biological molecules of relevance to antibiotic resistance. The small cross-hatched boxes represent the antibiotics and resistance genes of relevance to clinical practice. Respectively, these are a small subset of the world of small bioactive molecules (the parvome), and the world of potential resistance determinants (the resistome). The resistome comprises the genes that potentially encode resistance to antibiotics. The mobilome comprises the mobile proportion of bacterial genomes. The mobilome and resistome overlap, since many resistance genes are located on mobile elements. Both the resistome and mobilome are a subset of the total coding capacity of prokaryotic cells, the pangenome, which is expressed as the panproteome. Note that only a small proportion of the parvome is utilized by humans for antibiotic purposes, and that the scale of commercial antibiotic production probably overwhelms the natural production of these molecules by the entire global microbiota.

antibiotic resistance are ancient components of the pangenome, since they have been recovered from 30,000 year old permafrost (D'Costa et al., 2011), and from a cave microbiome that has been isolated for 4 million years (Bhullar et al., 2012). Note that the resistome overlaps with the mobilome (**Figure 2**), because many antibiotic resistance genes are found on mobile elements, allowing the resistome to be widely disseminated by LGT (Norman et al., 2009; Fondi and Fani, 2010; Skippington and Ragan, 2011). Also note that the resistance genes and mobile elements that are of concern for clinical antibiotic resistance (cross-hatched box, left hand side **Figure 2**) are just a fraction of the resistome and mobilome.

The **Parvome** is the world of small bioactive molecules produced by cells (also sometimes called secondary metabolites). The parvome includes important classes of molecules such as polyketides, aminoglycosides, terpenoids, alkaloids, and non-ribosomal peptides, many of which have antimicrobial activity (Davies, 2011; Davies and Ryan, 2011). The parvome overlaps with the proteome, because some of these small bioactive molecules are peptides. Note however, that only a small proportion of the parvome is utilized by humans for its antimicrobial properties (cross-hatched box, right hand side **Figure 2**). Commercial production of antibiotics overwhelms their natural synthesis, such that humans are now the major source of antibiotics in the general environment (**Figure 2**; Sarmah et al., 2006; Davies and Davies, 2010).

EVOLUTIONARY CONSEQUENCES OF THE ANTIBIOTIC AGE

Having examined the relationships between the key components of relevance to antibiotic production and resistance (**Figure 2**), we can now examine how each might be affected by human activity. There are several potential dimensions to these effects, including changes to the abundance and diversity of each component, and whether the effects are transient or permanent.

THE PARVOME

Antibiotics are released into the environment via human waste streams because a significant proportion of prophylactic antibiotics are excreted essentially unchanged (Sarmah et al., 2006; Le-Minh et al., 2010). Although it is difficult to obtain precise estimates for commercial production of antibiotics, it probably amounts to millions of metric tons per year (Segura et al., 2009). Because of the volume of antibiotics generated by human activity, the majority of the environmental load of antibiotics now originates from this commercial production (**Figure 2**; Davies and Davies, 2010). Certainly the local concentrations of this subset of the parvome have markedly increased due to human activity. Further, the diversity of small bioactive molecules has increased, because many antibiotics are synthetic modifications of natural structures. There is now a zone of influence around all human activities where the abundance and diversity of the parvome is increased. Such zones increase selective pressures on the local microbiome, not just the intended targets of antibiotic therapy (Martinez et al., 2009a; Taylor et al., 2011), and may also interfere with cell–cell communication.

THE RESISTOME

Increasing the concentration of antibiotics in the environment has effects on both the diversity and the abundance of genes belonging to the resistome. Selection for cells that carry resistance determinants increases their relative abundance, and thus increases the abundance of genes that confer resistance. Further, the ability of many elements of the resistome to undergo LGT, means that identical resistance genes can now be found in diverse bacterial species, from clinical contexts, from domestic animals, wild animals, and in locations apparently distant from the influence of developed societies, such as the Arctic, Antarctica, and the Amazonian jungle (Pallecchi et al., 2008; Sjolund et al., 2008; Bartoloni et al., 2009; Stokes and Gillings, 2011). That humans are responsible for this phenomenon is demonstrated by the positive correlation between the abundance of resistome elements of clinical significance and proximity to human activity (Skurnik et al., 2006; Hardwick et al., 2008; Thaller et al., 2010; Nardelli et al., 2012).

Selection pressure is particularly acute in human waste streams, where resistance genes are shed, mixed with high concentrations of antibiotics and other selective agents (Baquero et al., 2008; Schlüter et al., 2008; Moura et al., 2010). Exposure of environmental microorganisms to this mixture encourages fixation of LGT events, spreading elements of the resistome into diverse strains and species, thus further increasing their abundance, and their penetration into new hosts and niches.

Exposure to sub-inhibitory concentrations of antibiotics induces expression of error-prone DNA polymerases, increasing

the basal mutation rate (Kohanski et al., 2010; Thi et al., 2011). This has the effect of generating additional genomic diversity, and of co-opting additional genes into the resistome via mutational changes to housekeeping or accessory genes whose original function was not antibiotic resistance (Dantas and Sommer, 2012). As a second order effect, constant low level antibiotic exposure selects for lineages with inherently higher rates of mutation, generating additional diversity across the entire pangenome via drift and selection (Earl and Deem, 2004; Pigliucci, 2008; Couce and Blázquez, 2009; Gillings and Stokes, 2012).

Human activity has increased the abundance of resistome elements by selection, as shown by their increase in frequency in soils collected over the last 70 years (Knapp et al., 2009), and by their lateral transfer to diverse species. We have also increased the diversity and membership of the resistome by fixation of *de novo* mutations and by the co-option of genes as resistance determinants. Thus the size of the resistome, as a proportion of the pangenome, is probably becoming larger (**Figure 2**).

THE MOBILOME

The clinical use and environmental dissemination of antibiotics has had significant effects on the abundance and diversity of elements within the mobilome. Selection for antibiotic resistance has fixed lineages carrying diverse resistance determinants on a similarly diverse array of mobile elements. Such complex mobile elements now occur at high frequency in human-dominated systems, and in more natural ecosystems, where they can move by LGT into environmental organisms (Chee-Sanford et al., 2001; Nagachinta and Chen, 2008; Schlüter et al., 2008; Gillings et al., 2009a; Pellegrini et al., 2009).

The very use of antibiotics may itself increase the frequency of LGT, and thus the penetration of elements of the mobilome into new bacterial hosts (Beaber et al., 2004; Úbeda et al., 2005; Prudhomme et al., 2006). This effect is driven by the bacterial SOS response, which temporarily increases both the basal rate of LGT and of recombination (Tenaillon et al., 2004; Schlacher and Goodman, 2007). There is also evidence that human activities actually select for bacteria with a permanently increased propensity for LGT (Gillings and Stokes, 2012). Consequently, antibiotic pollution creates hotspots for the assembly of complex, mosaic mobile elements from diverse sources, and provides a selective force for their subsequent fixation in diverse lineages (Szczepanowski et al., 2005; Schlüter et al., 2008; Gillings et al., 2009b).

The accumulation of diverse mobile elements within single plasmids or at single loci provides opportunities for complex rearrangements and recombination events that in turn, generate more diversity (Garriss et al., 2009). The emergent properties that arise as mobile elements gain more components means that they can effectively increase their own complexity (Krizova et al., 2011; Toleman and Walsh, 2011). This phenomenon is evident in the increasing complexity and phenotypic plasticity of genomic islands and integrative conjugative elements in emerging nosocomial pathogens such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Fournier et al., 2006; Klockgether et al., 2011).

The selective forces acting upon complex mobile elements are not restricted to antibiotic pressure. Mobile DNAs often carry genes conferring resistance to other selective agents, such as heavy metals, arsenic, and disinfectants. Exposure to any one of these agents selects for lineages containing mobile elements with appropriate resistance genes, and simultaneously fixes all the other genes in physical linkage on that element, including antibiotic resistance determinants. Such co-selection of antibiotic resistance has long been thought to arise as a consequence of pollution with heavy metals (Baker-Austin et al., 2006; Salyers and Shoemaker, 2006; Stepanauskas et al., 2006; Wright et al., 2008; Rosewarne et al., 2010; Drudge et al., 2012). Disinfectant use also results in co-selection of antibiotic resistance (Gaze et al., 2005; Hegstad et al., 2010), and probably had a role in the origin of the class 1 integrons responsible for the widespread dissemination of antibiotic resistance amongst gram negative organisms (Gillings et al., 2008b).

High rates of LGT occur between organisms in similar Phyla, organisms with similar GC content, and organisms that occupy the same environmental niches (Thomas and Nielsen, 2005; Popa and Dagan, 2011). This activity creates lateral exchange communities, where DNA can be exchanged with relative ease (Beiko et al., 2005; Kloesges et al., 2010; Skippington and Ragan, 2011). Shared niches, and in particular, biofilms, promote such exchanges (Sorensen et al., 2005), and both conjugation and natural transformation appear to be important in the process (Kloesges et al., 2010; Domingues et al., 2012). While evolutionary distance and difference in GC content can restrict LGT, there are mechanisms for bypassing these barriers (Popa et al., 2011), and genes can make their way between distantly related organisms via a sequential series of lateral transfers.

Human use of antibiotics is therefore having multiple effects on the mobile components of the bacterial pangenome. One would predict that the size of the mobilome as a proportion of the pangenome is becoming larger, as more genes are recruited onto mobile elements (**Figure 2**). The number of resistome elements now residing on mobile DNA is also increasing, given that plasmids collected before the antibiotic era had no resistance determinants (Datta and Hughes, 1983; Hughes and Datta, 1983), and modern plasmids of similar structure have acquired a wide range of resistance genes dealing with various selective agents in addition to antibiotics. Consequently, there is now a greater overlap between the resistome and mobilome, particularly with respect to genes of concern for human health and welfare (**Figure 2**).

THE PANGENOME AND GLOBAL MICROBIOME

Human antibiotic use has pervasive effects on both the pangenome and the local composition of the microbiome, largely because antibiotics have non-target effects at gene, cell, and population levels. Exposure of cells to antibiotics induces an SOS response that has widespread effects on bacterial genomes, including raising the rates of mutation, recombination, and LGT (Tenaillon et al., 2004; Aertsen and Michiels, 2006; Schlacher and Goodman, 2007). This increase in basal rates of evolution applies to all the genes and cells in the exposed environment, not just the targets of antibiotic therapy. Consequently, the release of antibiotics into natural environments will affect the diversity of the pangenome and the composition and ecology of the global microbiota (Couce and Blázquez, 2009; Martinez, 2009a; Gillings and Stokes, 2012).

Effects in particular environments have been demonstrated experimentally. Short term antibiotic treatments lead to changes in abundance, richness, and diversity of the human gut microbiota (Dethlefsen et al., 2008), which can remain perturbed for years after treatment (Jakobsson et al., 2010; Sommer and Dantas, 2011). The composition and functional diversity of soil and sediment communities is affected by exposure to antibiotics (Kong et al., 2006; Cordova-Kreylos and Scow, 2007), and persistence of antibiotic residues in sediments or in the water column leads to alteration of microbiota and *in situ* selection for antibiotic resistance (Cabello, 2006; Knapp et al., 2008). While pulses of exposure to antibiotics may only produce transient selection events, alterations to community composition, and the fixation of resistance genes and their mobile vectors may be permanent, with unpredictable consequences for the whole microbiome (Martinez, 2009b; Gillings and Stokes, 2012).

ANTIBIOTICS AND RESISTANCE GENES AS POLLUTANTS

Antibiotics and their resistance genes originated from natural environments, but human use of antibiotics has perturbed the dynamics of this natural system. The high concentration of antibiotics used prophylactically by humans has two main consequences. Firstly, it leads to antibiotic contamination of human waste streams, and secondly, the selection imposed by antibiotic use has fixed ever more complex genetic elements in commensals and pathogens. These new "xenogenetic" elements are also released via human waste streams. While antibiotics might be treated as simple pollutants, xenogenetic mobile elements are capable of replication, and are thus more akin to invasive species (Gillings and Stokes, 2012).

Between 30 and 90% of the antibiotics given for human or veterinary use are excreted essentially unchanged (Sarmah et al., 2006). These compounds can be both persistent and mobile, and are often not removed during sewage treatment (Watkinson et al., 2007; Le-Minh et al., 2010; Zuccato et al., 2010). Antibiotics are also released in high concentrations from facilities where antibiotics are produced (Li et al., 2009, 2010), and are disseminated during application of manure to agricultural land (Chee-Sanford et al., 2009; Heuer et al., 2011). The consequences of pollution with antibiotics, particularly for aquatic systems, are being actively examined (Taylor et al., 2011; Lupo et al., 2012), and there is a call for development of policies to reduce the release of antibiotics and bacteria via human waste streams (Baquero et al., 2008).

Waste streams that release antibiotics into the environment also disseminate antibiotic resistance genes and mobile DNA elements, which should similarly be regarded as pollutants emanating from human activity (Pruden et al., 2006; Martinez, 2009a; Storteboom et al., 2010; Graham et al., 2011). The abundance of resistance genes in soils has been increasing since the introduction of antibiotics in the 1940s (Knapp et al., 2009), in parallel with the increasing concentrations of more conventional chemical pollutants.

Resistance genes and complex mobile elements are commonly reported from wastewater and sewage treatment plants (Tennstedt et al., 2003; Zhang et al., 2009; Pellegrini et al., 2011). Wastewater is regarded as a hotspot for interactions between mobile elements and for their lateral transfer between pathogens, commensals, and environmental bacteria (Schlüter et al., 2008; Moura et al., 2010). Resistome and mobilome elements are not necessarily removed by water treatment (Graham et al., 2011; Drudge et al., 2012), allowing resistance genes to be used as markers of human influence on aquatic ecosystems (Pei et al., 2006; Pruden et al., 2006; Storteboom et al., 2010).

Domestic and agricultural animals are a source of significant quantities of antibiotics and resistance genes. Animal waste and pig slurry are used to manure soils, with the consequent introduction of resistance genes and resistant bacteria (Binh et al., 2009; Byrne-Bailey et al., 2009; Gaze et al., 2011). The long term fate of antibiotics and resistance genes is difficult to predict without quantitative measurements over appropriate timescales (Chee-Sanford et al., 2009). However, it is clear that antibiotic resistant bacteria will increase in abundance, lateral transfers to soil organisms will occur, and resistance genes will be sequestered by diverse elements of the mobilome (Heuer et al., 2011).

CONCLUSION

Human use of antibiotics for medicine and agriculture may have consequences beyond their intended applications. Large quantities of antibiotics now emanate from human waste streams, as do the xenogenetic elements fixed in human ecosystems by antibiotic selection. Much more attention needs to be paid to the origins and fates of such pollutants (Allen et al., 2010). The antibiotic revolution may be having effects across the entire microbial biosphere (Martinez, 2009b), changing the basal rate of bacterial evolution, altering the composition of the resistome and mobilome, and promoting lateral transfer of mobile genetic elements (Couce and Blázquez, 2009; Gillings and Stokes, 2012). Antibiotic contamination promotes the fixation and mobilization of resistance genes between environmental and clinical microbiota (Kristiansson et al., 2011), and resistance genes are now widely spread through the biosphere (Martinez, 2009a; Stokes and Gillings, 2011).

Above all, we need to address the antibiotic resistance problem from a broader evolutionary and ecological perspective (Aminov and Mackie, 2007; Baquero et al., 2009; Fajardo et al., 2009). The ability of natural selection to shape species and communities is the same for microorganisms as it is for larger species (Gillings and Stokes, 2012), and the ecological theory of community assembly developed for multicellular organisms can be applied to the microbiome (Costello et al., 2012). The risk associated with the environmental spread of resistance genes with known adverse consequences for human welfare has had little attention, nor has the potential for pollution with antibiotics to widely affect the global microbiome. In comparison, the potential escape of resistance gene markers used in the generation of genetically modified plants has been the subject of considerable research (Martinez, 2012). It is time to pay more attention to the bioactive molecules that humans release into the environment.

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"Stormy waters ahead": global emergence of carbapenemases

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Carbapenems, once considered the last line of defense against of serious infections with Enterobacteriaceae, are threatened with extinction. The increasing isolation of carbapenem-resistant Gram-negative pathogens is forcing practitioners to rely on uncertain alternatives. As little as 5 years ago, reports of carbapenem resistance in Enterobacteriaceae, common causes of both community and healthcare-associated infections, were sporadic and primarily limited to case reports, tertiary care centers, intensive care units, and outbreak settings. Carbapenem resistance mediated by β-lactamases, or carbapenemases, has become widespread and with the paucity of reliable antimicrobials available or in development, international focus has shifted to early detection and infection control. However, as reports of *Klebsiella pneumoniae* carbapenemases, New Delhi metallo-β-lactamase-1, and more recently OXA-48 (oxacillinase-48) become more common and with the conveniences of travel, the assumption that infections with highly resistant Gram-negative pathogens are limited to the infirmed and the heavily antibiotic and healthcare exposed are quickly being dispelled. Herein, we provide a status report describing the increasing challenges clinicians are facing and forecast the "stormy waters" ahead.

Keywords: carbapenemases, NDM-1, KPC, OXA-48, metallo-β-lactamases, CHDL

Carbapenems are potent and broad-spectrum β-lactam antibiotics traditionally reserved for the treatment of the most serious infections (El-Gamal and Oh, 2010). The emergence and dissemination of carbapenem-resistant Gram-negative pathogens including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and Enterobacteriaceae is a significant contributor to patient morbidity and mortality (Patel et al., 2008; Schwaber et al., 2008; Lautenbach et al., 2009, 2010; Marchaim et al., 2011). Despite radical efforts in infection control (Schwaber et al., 2011) and improvements in rapid molecular diagnostics (Centers for Disease Control and Prevention, 2009; Nordmann et al., 2012c), carbapenem-resistant Gram-negative bacilli remain a formidable threat as few antimicrobial agents are reliably active and very little is expected to be available in the near future.

Clinicians hold that the increasing prevalence of extended-spectrum β-lactamases (ESBLs) among *Klebsiella pneumoniae* and *Escherichia coli* in the 1980s and 1990s contributed to the increased consumption of carbapenems. Experience implied that delayed administration of carbapenems in at-risk patients led to poor clinical outcomes (Paterson and Bonomo, 2005; Endimiani and Paterson, 2007). Thus, carbapenems (i.e., imipenem, meropenem, ertapenem, and doripenem) became vital tools in the treatment of healthcare-associated and severe community-acquired infections. Despite heavy reliance on these agents, carbapenem resistance in Enterobacteriaceae, common causes of both community and healthcare-associated infections, remained rare until the past decade.

Carbapenem resistance among Gram-negative bacteria results from one or more of the following mechanisms: (i) hyperproduction or derepression of Ambler class C β-lactamases (AmpC β-lactamases) or ESBLs (e.g., sulfhydryl variable (SHV), temoneira (TEM), cefotaxime (CTX-M) type β-lactamases) with loss or alteration in outer membrane porins; (ii) augmented drug efflux; (iii) alterations in penicillin binding proteins (PBPs); (iv) carbapenemase production (Patel and Bonomo, 2011). Carbapenemases belong to three molecular classes of β-lactamases, Ambler class A, B, and D (Ambler, 1980; Bush and Jacoby, 2010). Our aim is to provide a status report of the molecular diversity and epidemiology of carbapenemases as well as current and future therapeutics. The increasing public safety concerns associated with organisms harboring these enzymes has created significant turmoil. Regrettably, the situation is critical and our patients are in peril.

AMBLER CLASS A CARBAPENEMASES

Few Ambler class A β-lactamases demonstrate carbapenem-hydrolyzing activity and, up until a decade ago, these were rarely recovered. Class A carbapenemases include: *K. pneumoniae* carbapenemase (KPC), Guiana extended-spectrum (GES), non-metallo-carbapenemase-A (Nmc-A)/imipenem-resistant (IMI), *Serratia marcescens* enzyme (SME), serratia fonticola carbapenemase (SFC), and BIC β-lactamases (**Table 1**; Walther-Rasmussen and Høiby, 2007). With the notable exception of KPCs, the clinical isolation of these types of carbapenemases is relatively limited.

Table 1 | Class A carbapenemases*.

Enzyme	Year isolated or described	Organism(s)	Origin and geographic distribution	Location	Reference
Nmc-A	1990	*Enterobacter cloacae*	France, Argentina, USA	Chromosomal	Nordmann et al. (1993)
IMI-1	1984	*Enterobacter cloacae*	USA	Chromosomal	Rasmussen et al. (1996)
IMI-2	1999	*Enterobacter asburiae*, *Enterobacter cloacae*	USA†, China	Plasmid	Aubron et al. (2005), Yu et al. (2006)
SME-1	1982	*S. marcescens*	UK, USA	Chromosomal	Naas et al. (1994)
SME-2	1992	*S. marcescens*	USA, Canada, Switzerland	Chromosomal	Deshpande et al. (2006a), Poirel et al. (2007), Carrer et al. (2008)
SME-3	2003	*S. marcescens*	USA	Chromosomal	Queenan et al. (2006)
SFC-1	2003	*S. fonticola*	Portugal†	Chromosomal	Henriques et al. (2004)
GES-2	2000	*P. aeruginosa*	South Africa	Plasmid	Vourli et al. (2004)
GES-4	2002	*K. pneumoniae*	Japan	Plasmid	Wachino et al. (2004)
GES-5	2001	*K. pneumoniae*, *E. coli*, *P. aeruginosa*	Greece, Korea, worldwide	Plasmid	Jeong et al. (2005), Viau et al. (2012)
GES-6	2003	*K. pneumoniae*	Greece	Plasmid	Viau et al. (2012)
GES-11	2008	*Acinetobacter baumannii*	France	Plasmid	Moubareck et al. (2009)
GES-14	2010	*A. baumannii*	France	Plasmid	Bogaerts et al. (2010)
KPC-1‡	1996	*K. pneumoniae*	USA	Plasmid	Yigit et al. (2001)
KPC-2	1998	Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter* spp.	USA and worldwide	Plasmid§	Yigit et al. (2001)
KPC-3	2000	Enterobacteriaceae, *Acinetobacter* spp.	USA and worldwide	Plasmid	Woodford et al. (2004)
KPC-4	2003	*Enterobacter cancerogenus*, *K. pneumoniae*, *Acinetobacter* spp.	Scotland, Puerto Rico	Plasmid	Palepou et al. (2005), Robledo et al. (2007)
KPC-5	2006	*P. aeruginosa*	Puerto Rico	Plasmid	Wolter et al. (2009)
KPC-6	2003	*K. pneumoniae*	Puerto Rico	Plasmid	Bartual et al. (2005), Robledo et al. (2008)
KPC-7	2007	*K. pneumoniae*	USA	Plasmid	Perez et al. (2010a)
KPC-8	2008	*K. pneumoniae*	Puerto Rico	Plasmid	Diancourt et al. (2010)
KPC-9	2009	*E. coli*	Israel	Plasmid	Grosso et al. (2011)
KPC-10	2009	*Acinetobacter* spp.	Puerto Rico	Plasmid	Robledo et al. (2010)
KPC-11	2009	*K. pneumoniae*	Greece	Unknown	Da Silva et al. (2004)
KPC-12	2010	*E. coli*	China	Unknown	
KPC-13	2010	*Enterobacter cloacae*	Thailand	Unknown	
BIC-1	2009	*P. fluorescens*	France†	Chromosomal	Girlich et al. (2010)

** Adapted from Walther-Rasmussen and Høiby (2007).*
† *Environmental isolates.*
‡ *KPC-1 was later found to be the same enzyme as KPC-2 (Higgins et al., 2012a).*
§ *Chromosomal expression of* bla_{KPC-2} *has been described in P. aeruginosa (Villegas et al., 2007).*

Non-metallo-carbapenemase-A is a chromosomal carbapenemase originally isolated from *Enterobacter cloacae* in France (Nordmann et al., 1993). Currently, reports of this particular β-lactamase are still rare (Pottumarthy et al., 2003; Castanheira et al., 2008; Osterblad et al., 2012). IMI-1 was initially recovered from the chromosome of an *Enterobacter cloacae* isolate in the southwestern USA (Rasmussen et al., 1996). A variant of IMI-1, IMI-2, has been identified on plasmids isolated from environmental strains of *Enterobacter asburiae* in USA rivers (Aubron et al., 2005).

SME-1 (*S. marcescens* enzyme) was originally identified in an isolate of *S. marcescens* from a patient in London in 1982 (Yang et al., 1990). SME-2 and SME-3 were subsequently isolated in the USA, Canada, and Switzerland (Naas et al., 1994; Queenan et al., 2000, 2006; Deshpande et al., 2006b; Poirel et al., 2007; Carrer et al., 2008). Chromosomally encoded SME-type carbapenemases continue to be isolated at a low frequency in North America (Deshpande et al., 2006a,b; Fairfax et al., 2011; Mataseje et al., 2012). Both SFC-1 and BIC-1 are chromosomal serine carbapenemases recovered from environmental isolates. The former from

a *S. fonticola* isolate in Portugal (Henriques et al., 2004) and the latter from *Pseudomonas fluorescens* isolates recovered from the Seine River (Girlich et al., 2010).

The GES-type β-lactamases are acquired β-lactamases recovered from *P. aeruginosa*, Enterobacteriaceae, and *A. baumannii* (Poirel et al., 2000a; Castanheira et al., 2004a). The genes encoding these β-lactamase have often, but not exclusively, been identified within class 1 integrons residing on transferable plasmids (Bonnin et al., 2013; Walther-Rasmussen and Høiby, 2007). GES-1 has a similar hydrolysis profile to other ESBLs, although they essentially spare monobactams. Several GES β-lactamases are described with six (i.e., GES-2, GES-4, GES-5, GES-6, GES-11, and GES-14), demonstrating detectable carbapenemase activity in the setting of amino acid substitutions at their active sites (specifically at residue 104 and 170; Walther-Rasmussen and Høiby, 2007; Kotsakis et al., 2010). These GES-type carbapenemases have been described in Europe, South Africa, Asia, and the Middle East (Poirel et al., 2002; Jeong et al., 2005; da Fonseca et al., 2007; Moubareck et al., 2009; Bonnin et al., 2011, 2013).

Currently, most carbapenem resistance among Enterobacteriaceae in the USA and Israel is attributed to plasmid-mediated expression of a KPC-type carbapenemase (Endimiani et al., 2009b; Nordmann et al., 2009; Gupta et al., 2011; Schwaber et al., 2011). KPC-producing Enterobacteriaceae are considered endemic to Greece along with other carbapenemases, specifically VIM-type [Verona integron-encoded metallo-β-lactamases (MBLs); Canton et al., 2012]. KPCs efficiently hydrolyze carbapenems as well as penicillins, cephalosporins, and aztreonam and are not overcome *in vitro* by clinically available β-lactamase inhibitors (i.e., clavulanic acid, sulbactam, tazobactam – in fact these are hydrolyzed). These enzymes have been identified in several genera of Enterobacteriaceae as well as *Pseudomonas* spp. and *A. baumannii* (Miriagou et al., 2003; Yigit et al., 2003; Bratu et al., 2005; Villegas et al., 2007; Cai et al., 2008; Rasheed et al., 2008; Tibbetts et al., 2008; Robledo et al., 2010; Mathers et al., 2011; Geffen et al., 2012).

Carbapenem resistance secondary to KPC production was first described in a *K. pneumoniae* recovered in North Carolina in 1996 (Yigit et al., 2001). To date 12 KPC subtypes (KPC-2 to KPC-13; Robledo et al., 2008; Kitchel et al., 2009a; Navon-Venezia et al., 2009; Wolter et al., 2009; Gregory et al., 2010) have been reported with the vast majority of analyzed isolates expressing either KPC-2 or KPC-3.

The bla_{KPC} gene has been mapped to a highly conserved Tn*3*-based transposon, Tn*4401* (**Figure 1A**), and five isoforms of Tn*4401* are described (Naas et al., 2008; Cuzon et al., 2010; Kitchel et al., 2010). Plasmids carrying bla_{KPC} are of various sizes and many carry additional genes conferring resistance to fluoroquinolones and aminoglycosides thus limiting the antibiotics available to treat infections with KPC-producing pathogens (Endimiani et al., 2008; Rice et al., 2008). bla_{KPC} has rarely been mapped to a chromosomal location (Villegas et al., 2007; Castanheira et al., 2009).

A predominant strain of *K. pneumoniae* appears responsible for outbreaks and the international spread of KPC-producing *K. pneumoniae* (Woodford et al., 2008; Kitchel et al., 2009a; Samuelsen et al., 2009). Congruent pulsed-field gel electrophoresis (PFGE) patterns also suggest a clonal relationship between outbreak-associated strains of KPC-producing *K. pneumoniae* recovered from different areas that are endemic (Navon-Venezia et al., 2009; Woodford et al., 2011). The Centers for Disease Control and Prevention (CDC) performed PFGE and multilocus sequence typing (MLST) on isolates submitted to their reference laboratory from 1996 to 2008. A dominant PFGE pattern was observed and noted to be of a specific MLST type, ST 258 (Kitchel et al., 2009a). A second sequence type, ST 14, was common in institutions in the Midwest (Kitchel et al., 2009b). These findings implied that certain strains of *K. pneumoniae* may be more apt to obtain and retain the bla_{KPC} gene. Another study, however, analyzing 16 KPC-2 producing *K. pneumoniae* isolates from different geographic regions demonstrated diverse PFGE patterns and MLST types. This included four

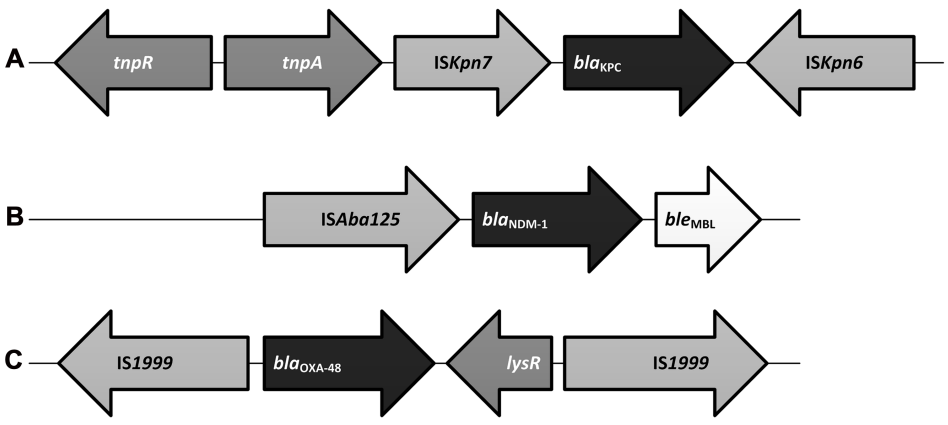

FIGURE 1 | Basic genetic construct of select carbapenemase genes. (A) Schematic representation of Tn*4401* type of transposon associated with bla_{KPC} which includes a transposase gene (*tnp*A), a resolvase gene (*tnp*R), as well as insertion sequences, IS*Kpn6* and IS*Kpn7* (Cuzon et al., 2010). **(B)** The bla_{NDM-1} construct demonstrates IS*Aba125* insertion sequence(s) upstream of the bla_{NDM-1} and a novel bleomycin resistance gene, ble_{MBL}, downstream (Dortet et al., 2012). **(C)** bla_{OXA-48} is often mapped to a Tn*1999* composite transposon where it is bracketed between two copies of the same insertion sequence, IS*1999*. Downstream of bla_{OXA-48} lies a *lysR* gene which encodes for a regulatory protein (Poirel et al., 2012b).

different MLST types in Colombia (ST 14, ST 337, ST 338, and ST 339) and two in Israel (ST 227 and ST 340). Although this study analyzed a smaller number of isolates, these findings suggest that the global propagation of KPC-2 is more complicated than the successful expansion of a fixed number of clones (Cuzon et al., 2010; Qi et al., 2011). More recently, a study evaluating the MLST types associated with widespread KPC-2 production in *K. pneumoniae* in Greece suggested that although ST 258 predominates at least 10 additional sequence types were found to carry bla_{KPC-2}. Of note three (i.e., ST 147, ST 323, and ST 383) carried both KPC-2 as well as genes encoding VIM-type MBLs (Giakkoupi et al., 2011; Woodford et al., 2011). A retrospective study in Cleveland documented the presence of ST 36 in a long-term care facility for children (Viau et al., 2012).

Klebsiella pneumoniae carbapenemases-production can confer variable levels of carbapenem resistance with reported minimum inhibitory concentrations (MICs) ranging from susceptible to ≥16 μg/mL. Analysis of isolates displaying high-level carbapenem resistance demonstrated that increased phenotypic resistance may be due to increased bla_{KPC} gene copy number or the loss of an outer membrane porin, OmpK35 and/or OmpK36. The highest level of imipenem resistance was seen with isolates lacking both porins and with augmented KPC enzyme production (Kitchel et al., 2010).

AMBLER CLASS B CARBAPENEMASES: METALLO-β-LACTAMASES

Class B β-lactamases (**Table 2**) are referred to as MBLs and require a metal ion, usually zinc, for β-lactam hydrolysis (Walsh et al., 2005). Due to the dependence on Zn^{2+}, catalysis is inhibited in the presence of metal-chelating agents like ethylenediaminetetraacetic acid (EDTA). MBL expression in Gram-negative bacteria confers resistance to penicillins, cephalosporins, and carbapenems. MBLs are not inhibited by the presence of commercially available β-lactamase inhibitors and susceptibility to monobactams (e.g., aztreonam) appears to be preserved in the absence of concomitant expression of other resistance mechanisms (e.g., ESBL production). The more geographically widespread MBLs include IMP (imipenem-resistant), VIM, and New Delhi metallo-β-lactamase (NDM).

Chromosomal MBLs were the first to be identified and are the cause of carbapenem resistance observed in *Bacillus cereus*, *Aeromonas* spp., and *Stenotrophomonas maltophilia* (Walsh et al., 2005). However, of growing concern are the "mobile" MBLs that have been reported since the mid-1990s. Although most frequently found in carbapenem-resistant isolates of *P. aeruginosa* and occasionally *Acinetobacter* spp., there is growing isolation of these enzymes in Enterobacteriaceae.

Prior to the description of NDM-1, frequently detected MBLs include IMP-type and VIM-type with VIM-2 being the most prevalent. These MBLs are embedded within a variety of genetic structures, most commonly integrons. When these integrons are associated with transposons or plasmids they can readily be transferred between species.

In 1991, IMP-1, a plasmid-mediated MBL, was identified in an isolates of *S. marcescens* from Japan (Ito et al., 1995). Since then plasmid-mediated carbapenem resistance secondary to IMP-1 spread widely in Japan, Europe, Brazil, and other parts of Asia and in several species of Gram-negative bacilli including *Acinetobacter* spp. and Enterobacteriaceae. At the present time, 42 variants of IMP have been identified with most cases of IMP-mediated carbapenem resistance being reported from Asia and among *P. aeruginosa* (Bush and Jacoby, 2010).

Table 2 | Metallo-β-lactamases.

Enzyme	Year isolated or described	Organism(s)	Geographic distribution	Location	Reference
IMP-1 to IMP-42	1988	Enterobacteriaceae, *Pseudomonas* spp., *Acinetobacter* spp.	Worldwide	Plasmid or chromosomal	Osano et al. (1994), Riccio et al. (2000)
VIM-1 to VIM-37	1997	Enterobacteriaceae, *Pseudomonas* spp., *Acinetobacter* spp.	Worldwide	Plasmid or chromosomal	Lauretti et al. (1999), Poirel et al. (2000b)
SPM-1	2001	*P. aeruginosa*	Brazil*	Chromosomal	Toleman et al. (2002)
GIM-1	2002	*P. aeruginosa*	Germany	Plasmid	Castanheira et al. (2004b)
SIM-1	2003–2004	*A. baumannii*	Korea	Chromosomal	Lee et al. (2005)
NDM-1 to NDM-7	2006	Enterobacteriaceae, *Acinetobacter* spp., *Vibrio cholerae*	Worldwide	Plasmid or chromosomal	Yong et al. (2009), Kaase et al. (2011), Nordmann et al. (2012a)
AIM-1	2007	*P. aeruginosa*	Australia	Chromosomal	Yong et al. (2007)
KHM-1	1997	*C. freundii*	Japan	Plasmid	Sekiguchi et al. (2008)
DIM-1	2007	*P. stutzeri*	Netherlands	Plasmid	Poirel et al. (2010c)
SMB-1	2010	*S. marcescens*	Japan	Chromosomal	Wachino et al. (2011)
TMB-1	2011	*Achromobacter xylosoxidans*	Libya	Chromosomal	El Salabi et al. (2012)
FIM-1	2007	*P. aeruginosa*	Italy	Chromosomal	Pollini et al. (2012)

**Single report of SPM-1 in Europe linked to healthcare exposure in Brazil (Salabi et al., 2010).*

A more commonly recovered MBL is the VIM-type enzyme. VIM-1 was first described in Italy in 1997 in *P. aeruginosa* (Lauretti et al., 1999). VIM-2 was next discovered in southern France in *P. aeruginosa* cultured from a neutropenic patient in 1996 (Poirel et al., 2000b). Although originally thought to be limited to non-fermenting Gram-negative bacilli, VIM-type MBLs are being increasingly identified in Enterobacteriaceae as well (Giakkoupi et al., 2003; Kassis-Chikhani et al., 2006; Morfin-Otero et al., 2009; Canton et al., 2012). To date, 37 variants of VIM have been described with VIM-2 being the most common MBL recovered worldwide.

Other more geographically restricted MBLs include SPM-1 (Sao Paulo MBL), which has been associated with hospital outbreaks in Brazil (Toleman et al., 2002; Rossi, 2011); GIM-1 (German imipenemase) isolated in carbapenem-resistant *P. aeruginosa* isolates in Germany (Castanheira et al., 2004b); SIM-1 (Seoul imipenemase) isolated from *A. baumannii* isolates in Korea (Lee et al., 2005); KHM-1 (Kyorin Health Science MBL) isolated from a *C. freundii* isolate in Japan (Sekiguchi et al., 2008); AIM-1 (Australian imipenemase) isolated from *P. aeruginosa* in Australia (Yong et al., 2007); DIM-1 (Dutch imipenemase) isolated from a clinical *P. stutzeri* isolate in the Netherlands (Poirel et al., 2010c); SMB-1 (*S. marcescens* MBL) in *S. marcescens* in Japan (Wachino et al., 2011); TMB-1 (Tripoli MBL) in *Achromobacter xylosoxidans* in Libya (El Salabi et al., 2012), and FIM-1 (Florence imipenemase) from a clinical isolate of *P. aeruginosa* in Italy (Pollini et al., 2012). With the notable exception of SPM-1, these MBLs have remained confined to their countries of origin (Salabi et al., 2010).

NDM-1 was first identified in 2008. Due to its rapid international dissemination and its ability to be expressed by numerous Gram-negative pathogens, NDM is poised to become the most commonly isolated and distributed carbapenemase worldwide. Initial reports frequently demonstrated an epidemiologic link to the Indian subcontinent where these MBLs are endemic (Kumarasamy et al., 2010). Indeed, retrospective analyses of stored isolates suggest that NDM-1 may have been circulating in the subcontinent as early as 2006 (Castanheira et al., 2011). Despite initial controversy, the Balkans may be another area of endemicity for NDM-1 (Struelens et al., 2010; Jovcic et al., 2011; Livermore et al., 2011c; Halaby et al., 2012). Sporadic recovery of NDM-1 in the Middle East suggests that this region may be an additional reservoir (Poirel et al., 2010a, 2011d; Nordmann et al., 2011; Ghazawi et al., 2012).

Like KPCs, the conveniences of international travel and medical tourism have quickly propelled this relatively novel MBL into a formidable public health threat. Gram-negative bacilli harboring bla_{NDM} have been identified worldwide with the exception of Central and South America.

NDM-1 was first identified in Sweden in a patient of Indian descent previously hospitalized in India (Yong et al., 2009). The patient was colonized with a *K. pneumoniae* and an *E. coli* carrying $bla_{\mathrm{NDM-1}}$ on transferable plasmids. In the UK, an increase in the number of clinical isolates of carbapenem-resistant Enterobacteriaceae was seen in both 2008 and 2009. A UK reference laboratory reported that at least 17 of 29 patients found to be harboring NDM-1 expressing Enterobacteriaceae had a history of recent travel to the Indian subcontinent with the majority having been hospitalized in those countries (Kumarasamy et al., 2010).

European reports suggest that horizontal transfer of $bla_{\mathrm{NDM-1}}$ exists within hospitals outside endemic areas. Of overwhelming concern are the reported cases without specific contact with the healthcare system locally or in endemic areas suggesting autochthonous acquisition (Kumarasamy et al., 2010; Kus et al., 2011; Arpin et al., 2012; Borgia et al., 2012; Nordmann et al., 2012b).

Surveillance of public water supplies in India indicates that exposure to NDM-1 may be environmental. Walsh et al. (2011) analyzed samples of public tap water and seepage water from sites around New Delhi. The results were disheartening in that $bla_{\mathrm{NDM-1}}$ was detected by PCR in 4% of drinking water samples and 30% of seepage samples. In this survey, carriage of $bla_{\mathrm{NDM-1}}$ was noted in 11 species of bacteria not previously described, including virulent ones like *Shigella boydii* and *Vibrio cholerae*.

The rapid spread of NDM-1 highlights the fluidity and rapidity of gene transfer between bacterial species. Although $bla_{\mathrm{NDM-1}}$ was initially and repeatedly mapped to plasmids isolated from carbapenem-resistant *E. coli* and *K. pneumoniae*, reports of both plasmid and chromosomal expression of $bla_{\mathrm{NDM-1}}$ has been noted in other species of Enterobacteriaceae as well as *Acinetobacter* spp. and *P. aeruginosa* (Moubareck et al., 2009; Bogaerts et al., 2010; Bonnin et al., 2011; Nordmann et al., 2011; Patel and Bonomo, 2011). Recently, bacteremia with a NDM-1 expressing *V. cholerae* has been described in a patient previously hospitalized in India colonized with a variety of Enterobacteriaceae previously known to be capable of carrying plasmids with $bla_{\mathrm{NDM-1}}$ (Darley et al., 2012).

In contrast to KPCs, the presence of a dominant clone among $bla_{\mathrm{NDM-1}}$ carrying isolates remains elusive (Poirel et al., 2011c). NDM-1 expression in *E. coli* has been noted among sequence types previously associated with the successful dissemination of other β-lactamases including ST 101 and ST 131 (Mushtaq et al., 2011). Mushtaq et al. (2011) analyzed a relatively large group of $bla_{\mathrm{NDM-1}}$ expressing *E. coli* from the UK, Pakistan, and India in order to potentially identify a predominant strain responsible for the rapid and successful spread of NDM-1. The most frequent sequence type identified was ST 101. Another study examining a collection of carbapenem-resistant Enterobacteriaceae from India demonstrates the diversity of strains capable of harboring $bla_{\mathrm{NDM-1}}$. Carriage of $bla_{\mathrm{NDM-1}}$ was confirmed in 10 different sequence types of *K. pneumoniae* and 5 sequence types of *E. coli* (Lascols et al., 2011). This multiplicity was confirmed in a study looking at a collection of $bla_{\mathrm{NDM-1}}$ expressing Enterobacteriaceae from around the world (Poirel et al., 2011c). Of most concern is that NDM-1 has been identified in *E. coli* ST 131, the strain of *E. coli* credited with the global propagation of CTX-M-15 ESBLs (Mushtaq et al., 2011; Peirano et al., 2011; Pfeifer et al., 2011b; Woodford et al., 2011). Similar to KPCs, NDM-1 expression portends variable levels of carbapenem resistance and there is often concomitant carriage of a myriad of resistance determinants including other β-lactamases and carbapenemases as well as genes associated with resistance to fluoroquinolones and aminoglycosides (Nordmann et al., 2011).

NDM-1 shares the most homology with VIM-1 and VIM-2. It is a 28-kDa monomeric protein that demonstrates tight binding to both penicillins and cephalosporins (Zhang and Hao, 2011). Binding to carbapenems does not appear to be as strong as other MBLs, but hydrolysis rates appear to be similar. Using ampicillin as a substrate, allowed for detailed characterization of the interactions between NDM's active site and β-lactams as well as improved evaluation of MBLs unique mechanism of β-lactam hydrolysis. More recent crystal structures of NDM-1 reveal the molecular details of how carbapenem antibiotics are recognized by dizinc-containing MBLs (King et al., 2012).

To date, NDM-1 remains the most common NDM variant isolated. Seven variants (NDM-1 to NDM-7) exist (Kaase et al., 2011; Nordmann et al., 2012a). It is currently held that bla_{NDM-1} is a chimeric gene that may have evolved from *A. baumannii* (Toleman et al., 2012). Contributing to this theory is the presence of complete or variations of the insertion sequence, IS*Aba125*, upstream to the bla_{NDM-1} gene in both Enterobacteriaceae and *A. baumannii* (Pfeifer et al., 2011a; Poirel et al., 2011a; Dortet et al., 2012; Toleman et al., 2012). This insertion sequence has primarily been found in *A. baumannii*.

A recent evaluation of the genetic construct associated with bla_{NDM-1} (**Figure 1B**) has lead to the discovery of a new bleomycin resistance protein, BRP_{MBL}. Evaluation of 23 isolates of $bla_{NDM-1/2}$ harboring Enterobacteriaceae and *A. baumannii* noted that the overwhelming majority of them possessed a novel bleomycin resistance gene, ble_{MBL} (Dortet et al., 2012). Co-expression of bla_{NDM-1} and ble_{MBL} appear to be mediated by a common promoter (P_{NDM-1}) which includes portions of IS*Aba125*. It is postulated that BRP_{MBL} expression may contribute some sort of selective advantage allowing NDM-1 to persist in the environment.

A contemporary evaluation of recently recovered NDM-1 producing *A. baumannii* isolates from Europe demonstrates that bla_{NDM-1} and bla_{NDM-2} genes are situated on the same chromosomally located transposon, Tn*125* (Bonnin et al., 2012). Dissemination of bla_{NDM} in *A. baumannii* seems be due to different strains carrying Tn*125* or derivatives of Tn*125* rather than plasmid-mediated or clonal (Bonnin et al., 2013; Poirel et al., 2012a).

CARBAPENEM-HYDROLYZING CLASS D β-LACTAMASES

Oxacillinases comprise a heterogeneous group of class D β-lactamases which are able to hydrolyze amino- and carboxypenicillins (Poirel et al., 2010b). The majority of class D β-lactamases are not inhibited by commercially available β-lactamase inhibitors but are inhibited *in vitro* by NaCl. Over 250 types of oxacillinases are reported with a minority demonstrating low levels of carbapenem-hydrolyzing activity. This select group of enzymes is also referred to as the carbapenem-hydrolyzing class D β-lactamases (CHDLs; **Table 3**). CHDLs have been identified most frequently in *Acinetobacter* spp., however, there has been increasing isolation among Enterobacteriaceae, specifically OXA-48 (oxacillinase-48; Lascols et al., 2012; Mathers et al., 2012).

With the exception of OXA-163 (Poirel et al., 2011b), CHDLs efficiently inactivate penicillins, first generations cephalosporins, and β-lactam/β-lactamase inhibitor combinations, but spare extended-spectrum cephalosporins. Carbapenem hydrolysis efficiency is lower than that of other carbapenemases, including the MBLs, and often additional resistance mechanisms are expressed in organisms demonstrating higher levels of phenotypic carbapenem resistance. These include expression of other carbapenemases, alterations in outer membrane proteins (e.g., CarO, OmpK36; Perez et al., 2007; Gülmez et al., 2008; Pfeifer et al., 2012), increased transcription mediated by *IS* elements functioning as promoters, increased gene copy number, and amplified drug efflux (Poirel and Nordmann, 2006; Perez et al., 2007). Many subgroups of CHDLs have been described. We will focus on those found in *A. baumannii* and Enterobacteriaceae: OXA-23 and OXA-27; OXA-24/40, OXA-25, and OXA-26; OXA-48 variants; OXA-51, OXA-66, OXA-69; OXA-58, and OXA-143.

CHDLs can be intrinsic or acquired. *A. baumannii* does have naturally occurring but variably expressed chromosomal CHDLs, OXA-51, OXA-66, and OXA-69 (Brown et al., 2005; Héritier et al., 2005b). For the most part, in isolation the phenotypic carbapenem resistance associated with these oxacillinases is low. However, levels of carbapenem resistance appear to be increased in the presence of specific insertion sequences promoting gene expression (Figueiredo et al., 2009; Culebras et al., 2010). Additional resistance to extended-spectrum cephalosporins can be seen in the setting of co-expression of ESBLs and/or other carbapenemases (Castanheira et al., 2011; Mathers et al., 2012; Pfeifer et al., 2012; Voulgari et al., 2012; Potron et al., 2013).

The first reported "acquired" oxacillinase with appreciable carbapenem-hydrolyzing activity was OXA-23. OXA-23, or ARI-1, was identified from an *A. baumannii* isolate in Scotland in 1993 (the isolate was first recovered in 1985; Paton et al., 1993). Subsequently, OXA-23 expression has been reported worldwide (Mugnier et al., 2010) and both plasmid and chromosomal carriage of bla_{OXA-23} are described. The OXA-23 group includes OXA-27, found in a single *A. baumannii* isolate from Singapore (Afzal-Shah et al., 2001). With the exception of an isolate of *Proteus mirabilis* identified in France in 2002, this group of β-lactamases has been exclusively recovered from *Acinetobacter* species (Bonnet et al., 2002). Increased expression of OXA-23 has been associated with the presence of upstream insertion sequences (e.g., IS*Aba1* and IS*Aba4*) acting as strong promoters (Corvec et al., 2007).

Another group of CHDLs include OXA-24/40, OXA-25, and OXA-26 (Bou et al., 2000b; Afzal-Shah et al., 2001). OXA-24 and OXA-40 differ by a few amino acid substitutions and OXA-25 and OXA-26 are point mutation derivatives of OXA-40 (Afzal-Shah et al., 2001). Although primarily linked with clonal outbreaks in Spain and Portugal (Bou et al., 2000a; Lopez-Otsoa et al., 2002; Da Silva et al., 2004; Acosta et al., 2011), OXA-24/40 β-lactamases has been isolated in other European countries and the USA (Lolans et al., 2006). OXA-40 was in fact the first CHDL documented in the USA (Lolans et al., 2006).

OXA-58 has also only been detected in *Acinetobacter* spp. initially identified in France (Héritier et al., 2005a; Poirel et al., 2005), OXA-58 has been associated with institutional outbreaks and has been recovered from clinical isolates of *A. baumannii* worldwide (Coelho et al., 2006; Mendes et al., 2009; Gales et al., 2012).

As civilian and military personnel began returning from Afghanistan and the Middle East, practitioners noted increasing

Table 3 | Carbapenem-hydrolyzing class D β-lactamases.

Enzyme group	Year isolated or described	Organism(s)	Geographic distribution	Location	Reference
OXA-23/27	1985/–	*Acinetobacter baumannii*, *Proteus mirabilis**	Europe, USA, Middle East, Asia, Australia	Plasmid, chromosomal	Afzal-Shah et al. (2001), Gogou et al. (2011)
OXA-24/40	1997	*A. baumannii*	Europe and USA	Plasmid, chromosomal	Bou et al. (2000b), Lopez-Otsoa et al. (2002)
OXA-25	–	*A. baumannii*	Spain	Chromosomal	Afzal-Shah et al. (2001)
OXA-26	1996	*A. baumannii*	Belgium	Chromosomal	Afzal-Shah et al. (2001)
OXA-48	2001	*K. pneumoniae*, Enterobacteriaceae	Turkey, Middle East, Northern Africa, Europe, India, USA	Plasmid	Poirel et al. (2004b)
OXA-51/66/69	1993	*A. baumannii*	Worldwide	Chromosomal	Brown et al. (2005), Evans et al. (2007)
OXA-58	2003	*A. baumannii*	Europe, USA, Middle East, South America	Plasmid	Poirel et al. (2005)
OXA-143	2004	*A. baumannii*	Brazil	Plasmid	Higgins et al. (2009)
OXA-162	2008	Enterobacteriaceae	Germany	Plasmid	Pfeifer et al. (2012)
OXA-163	2008	*K. pneumoniae*, *E. coli*	Argentina and Egypt	Plasmid	Poirel et al. (2011b), Abdelaziz et al. (2012)
OXA-181	2006	*K. pneumoniae*, *E. coli*	India	Plasmid	Castanheira et al. (2011)
OXA-204	2012	*K. pneumoniae*	Tunisia	Plasmid	Potron et al. (2013)
OXA-232	2012	*K. pneumoniae*	France	Plasmid	Poirel et al. (2012c)

**Single isolate described in France.*

recovery of *A. baumannii* from skin and soft tissue infections. Drug resistance was associated with expression of both OXA-23 and OXA-58 (Hujer et al., 2006; Scott et al., 2007; Perez et al., 2010b). Many isolates carrying the bla_{OXA-58} gene concurrently carry insertion sequences (e.g., IS*Aba1*, IS*Aba2*, or IS*Aba3*) associated with increased carbapenemase production and thus higher levels of carbapenem resistance. In one report increased gene copy number was also associated with a higher level of enzyme production and increased phenotypic carbapenem resistance (Bertini et al., 2007).

Spread of OXA-type carbapenemases among *A. baumannii* appears to be clonal and in depth reviews of the molecular epidemiology and successful dissemination of these clones have been published (Woodford et al., 2011; Zarrilli et al., 2013). Two MLST schemes with three loci in common exist for *A. baumannii* – the PubMLST scheme (Bartual et al., 2005) and the Pasteur scheme (Diancourt et al., 2010). Both schemes assign different sequence types into clonal complexes (CC). Sequence types and CC from both schemes can be further categorized into the international (European) clones I, II, and III. It should be noted, however, that the molecular taxonomy of *A. baumannii* continues to evolve (Higgins et al., 2012a). OXA-23 producing *A. baumannii* predominantly belong to international clones I and II with a notable proportion being part of CC92 (PubMLST; Mugnier et al., 2010; Adams-Haduch et al., 2011). Similarly, *A. baumannii* isolates associated with epidemic spread of OXA-24/40 in Portugal and Spain appear are incorporated in international clone II (Da Silva et al., 2004; Grosso et al., 2011) and ST 56 (PubMLST; Acosta et al., 2011). OXA-58 expressing *A. baumannii* have been associated with international clones I, II, and II and a variety of unrelated sequence types (Di Popolo et al., 2011; Gogou et al., 2011).

OXA-48 was originally identified in a carbapenem-resistant isolate of *K. pneumoniae* in Turkey (Poirel et al., 2004c). Early reports suggested that this enzyme was geographically restricted to Turkey. In the past few years, however, the enzyme has been recovered from variety of Enterobacteriaceae and has successfully circulated outside of Turkey with reports of isolation in the Middle East, North Africa, Europe (Carrer et al., 2010), and most recently the USA (Lascols et al., 2012; Mathers et al., 2012). The Middle East and North Africa may be secondary reservoirs for these CHDLs (Hays et al., 2012; Poirel et al., 2012c). Indeed, the introduction of OXA-48 expressing Enterobacteriaceae in some countries has been from patients from the Middle East or Northern Africa (Decre et al., 2010; Adler et al., 2011; Poirel et al., 2011e; Canton et al., 2012). In the USA, the first clinical cases were associated with ST 199 and ST 43 (Mathers et al., 2012).

At least six OXA-48 variants (e.g., OXA-48, OXA-162, OXA-163, OXA-181, OXA-204, and OXA-232) have been identified. OXA-48 is by far the most globally dispersed and its epidemiology has been recently reviewed (Poirel et al., 2012c). Unlike KPCs and NDM-1 which have been associated with a variety of plasmids, a

single 62 kb self-conjugative IncL/M-type plasmid has contributed to a large proportion of the distribution of bla_{OXA-48} in Europe (Potron et al., 2011a). Sequencing of this plasmid (pOXA-48a) notes that bla_{OXA-48} had been integrated through the acquisition of a Tn*1999* composite transposon (**Figure 1C**; Poirel et al., 2012b) bla_{OXA-48} appears to be associated with a specific insertion sequence, IS*1999* (Poirel et al., 2004c, 2012b). A variant of Tn*1999*, Tn*1999.2*, has been identified among isolates from Turkey and in Europe (Carrer et al., 2010; Potron et al., 2011a). Tn*1999.2* harbors an IS*1R* element within the IS*1999*. OXA-48 appears to have the highest affinity for imipenem of the CHDLs specifically those harboring bla_{OXA-48} within a Tn*1999.2* composite transposon (Docquier et al., 2009). Three isoforms of the Tn*1999* transposon have been described (Giani et al., 2012).

Although much of the spread of OXA-48 is attributed to a specific plasmid, outbreak evaluations demonstrate that a variety of strains have contributed to dissemination of this emerging carbapenemase in *K. pneumoniae*. The same *K. pneumoniae* sequence type, ST 395, harboring bla_{OXA-48} was identified in Morocco, France, and the Netherlands (Cuzon et al., 2011; Potron et al., 2011a). ST 353 was associated with an outbreak of OXA-48 producing *K. pneumoniae* in London (Woodford et al., 2011) and ST 221 with an outbreak of OXA-48 in Ireland (Canton et al., 2012). OXA-48 production in *K. pneumoniae*, like KPC-expressing *K. pneumoniae*, has also been associated with ST 14 (Poirel et al., 2004c) and a recent outbreak in Greece was associated with ST 11 (Voulgari et al., 2012).

bla_{OXA-48} is remarkably similar to bla_{OXA-54}, a β-lactamase gene intrinsic to *Shewanella oneidensis* (Poirel et al., 2004a). *Shewanella* spp. are relatively ubiquitous waterborne Gram-negative bacilli and are proving to be a potential environmental reservoir for OXA-48 like carbapenemases as well as other resistance determinants (Héritier et al., 2004; Poirel et al., 2004b; Potron et al., 2011b).

OXA-163, a single amino acid variant of OXA-48, was identified in isolates of *K. pneumoniae* and *Enterobacter cloacae* from Argentina and is unique in that it has activity against extended-spectrum cephalosporins (Poirel et al., 2011b). OXA-163 also has been identified in Egypt, which has a relatively prevalence of OXA-48, in patients without epidemiologic links to Argentina (Abdelaziz et al., 2012).

OXA-181 was initially identified among carbapenem-resistant Enterobacteriaceae collected from India (Castanheira et al., 2011). OXA-181 differs from OXA-48 by four amino acids, however, appears to be nestled in an entirely different genetic platform. The $bla_{OXA-181}$ gene has been mapped to a different group of plasmids, the ColE family, and has been associated with an alternative insertion sequence, IS*Ecp1*. The latter insertion sequence has been associated with the acquisition of other β-lactamases including CTX-M-like ESBLs. Like, OXA-48, it appears that OXA-181 may have evolved from a waterborne environmental species *Shewanella xiamenensis* (Potron et al., 2011b).

OXA-204 differs from OXA-48 by a two amino acid substitution. It was recently identified in a clinical *K. pneumoniae* isolate from Tunisia (Potron et al., 2013). Its genetic construct appears to be similar to that of OXA-181. OXA-232 was recently identified among *K. pneumoniae* isolates in France (Poirel et al., 2012c).

OXA-143 is a novel plasmid-borne carbapenem-hydrolyzing oxacillinase recovered from clinical *A. baumannii* isolates in Brazil (Higgins et al., 2009). Information regarding its significance and prevalence continues to evolve (Antonio et al., 2010; Werneck et al., 2011; Mostachio et al., 2012).

AVAILABLE AGENTS AND DRUGS IN DEVELOPMENT

Few antimicrobials are currently available to treat infections with carbapenemase-producing Gram-negative bacteria. Carriage of concurrent resistance determinants can result in decreased susceptibility non-β-lactams including the fluoroquinolones and aminoglycosides thus further compromising an already limited antimicrobial arsenal. What frequently remains available are the polymyxins (including colistin), tigecycline, and fosfomycin but susceptibilities to these agents are unpredictable (Falagas et al., 2011).

The reintroduction of polymyxins, both polymyxin B and colistin overlaps with the evolution of carbapenem resistance among Gram-negative bacilli. The clinical "resurgence" of these agents is well documented (Falagas and Kasiakou, 2005; Li et al., 2006a; Landman et al., 2008). Some experts advocate for the use of polymyxins in combination with other agents like rifampicin (Hirsch and Tam, 2010; Urban et al., 2010). *In vitro* evaluations of different combinations including carbapenems, rifamycins, and/or tigecycline demonstrate variable results (Bercot et al., 2011; Biswas et al., 2012; Deris et al., 2012; Jernigan et al., 2012). Most evaluations of the clinical outcomes or "effectiveness" of combination therapies have been retrospective (Qureshi et al., 2012; Tumbarello et al., 2012). Prospective clinical trials evaluating the superiority of colistin-based combination therapy over monotherapy are in their infancy. A real interest in combination therapy persists due to the concern of hetero-resistance (Li et al., 2006b; Poudyal et al., 2008; Lee et al., 2009; Yau et al., 2009; Meletis et al., 2011).

Early evaluations of the glycylcycline, tigecycline, demonstrated favorable *in vitro* activity against ESBL-producing Enterobacteriaceae and specific isolates of carbapenem-resistant *A. baumannii* and Enterobacteriaceae (Bratu et al., 2005; Fritsche et al., 2005; Noskin, 2005; Castanheira et al., 2008; Wang and Dowzicky, 2010). Tigecycline remains untested in prospective trials and reports of resistance are increasing (Navon-Venezia et al., 2007; Anthony et al., 2008; Wang and Dowzicky, 2010; Sun et al., 2012). The role of tigecycline in treating primary bloodstream infections or urinary tract infections remains undefined due less than therapeutic concentrations of drug achieved in the serum (Rodvold et al., 2006) and urine (Satlin et al., 2011). We also note that meta-analyses of pooled data from trials evaluating the use of tigecycline for a variety of indications suggest there is a excess mortality associated with the use of tigecycline over comparator regimens (Cai et al., 2011; Tasina et al., 2011; Yahav et al., 2011; Verde and Curcio, 2012). However, in the absence of other tested regimens tigecycline may be an appropriate or perhaps the only therapeutic option.

Growing resistance to both the polymyxins and tigecycline has resulted the revisiting of older drugs including chloramphenicol, nitrofurantoin, and temocillin (Livermore et al., 2011d). Fosfomycin is also one of these earlier antibiotics being reassessed

(Falagas et al., 2008). In an *in vitro* evaluation of 68 KPC-expressing *K. pneumoniae* isolates, fosfomycin demonstrated *in vitro* activity against 87% of tigecycline and/or polymyxin non-susceptible isolates and 83% of isolates that were resistant to both (Endimiani et al., 2010b). Fosfomycin may be a potential therapeutic option for patients infected with carbapenemase-producing Enterobacteriaceae if the infection is localized to the genitourinary tract. Unfortunately, fosfomycin does not demonstrate reliable activity against non-urinary pathogens. Fosfomycin demonstrated activity against only 30.2% of 1693 multidrug-resistant (MDR) *P. aeruginosa* isolates and 3.5% of 85 MDR *A. baumannii* isolates (Falagas et al., 2009). The individual studies included in this review did not employ uniform MDR definitions or consistent susceptibility breakpoints. Moreover, access to the parenteral fosfomycin is limited and the threshold for resistance is low (Rodriguez-Rojas et al., 2010; Karageorgopoulos et al., 2012). Concerns regarding the emergence of resistance have lead to an increasing interest in the utility of combination therapy (Michalopoulos et al., 2010; Bercot et al., 2011; Souli et al., 2011).

Few agents are in the advanced stages of development with demonstrable *in vitro* activity against carbapenemase-producing organisms. These include β-lactamase inhibitors, aminoglycoside derivatives, polymyxin derivatives, and novel monobactams and monobactams-β-lactamase inhibitor combinations.

Avibactam, or NXL104, is a β-lactamase inhibitor which has been tested in combination with ceftazidime, ceftaroline, and aztreonam against several carbapenemase-producing Enterobacteriaceae with impressive decreases in MICs (Livermore et al., 2008, 2011b; Endimiani et al., 2009a; Mushtaq et al., 2010c). Cephalosporin–avibactam combinations do not inhibit MBLs. Avibactam in combination with aztreonam, however, does seem to demonstrate activity against isolates harboring a variety of carbapenem resistance mechanisms including MBLs (Livermore et al., 2011b). Regrettably, the avibactam and aztreonam combination is not currently in clinical trials. The combination of ceftazidime–avibactam has been evaluated against collections of non-fermenting Gram-negative pathogens and its role remains undefined (Mushtaq et al., 2010b). In some evaluations of ceftazidime non-susceptible isolates of *P. aeruginosa* decrease MICs were noted with the addition of avibactam (Mushtaq et al., 2010b; Walkty et al., 2011; Crandon et al., 2012; Levasseur et al., 2012). The combinations of ceftaroline–avibactam and ceftazidime–avibactam are currently in clinical trials.

Methylidene penems (penem-1 and penem-2) are β-lactamase inhibitors and appear to be potent inhibitors of KPC-2 (Papp-Wallace et al., 2010). The combination of cefepime with penem-1 demonstrated lower cefepime MICs in 88.1% of the 42 KPC-producing *K. pneumoniae* isolates evaluated (Endimiani et al., 2010a). MK-7655 is a novel β-lactamase being evaluated in combination with imipenem against carbapenem-resistant Gram-negative bacilli (Hirsch et al., 2012).

ME1071, formerly CP3242 (Bassetti et al., 2011), is a maleic acid derivative that competitively inhibits MBLs. Earlier studies demonstrated concentration-dependent decreases in carbapenem MICs in MBL-producing *P. aeruginosa* (Ishii et al., 2010), *A. baumannii*, and select Enterobacteriaceae (Shahid et al., 2009) A contemporary pre-clinical evaluation of ME1071 in combination with various type 2 carbapenems (i.e., biapenem, doripenem, meropenem, imipenem) confirms remarkable decreases in the carbapenem MICs for Enterobacteriaceae and *A. baumannii* harboring IMP, VIM, and NDM-type MBLs (Livermore et al., 2013). Irrespective of the candidate carbapenem, ME1071 activity against NDM MBLs was less than that of VIM-type and IMP-type MBLs. Of note, biapenem was the carbapenem with the lowest baseline MICs to the MBLs, but it is commercially unavailable in many countries including the USA. Other MBL-specific inhibitors are in pre-clinical development (Chen et al., 2012).

Plazomicin (ACHN-490) is an aminoglycoside derivative with potent activity against some carbapenem-resistant Gram-negative bacilli (Zhanel et al., 2012). Studies have noted that susceptibilities to aminoglycosides vary among KPC-producing *K. pneumoniae*. In one evaluation, 48% of 25 tested isolates were susceptible to amikacin, 44% to gentamicin, and 8% to tobramycin. Plazomicin demonstrated an MIC_{90} significantly lower than that of amikacin (Endimiani et al., 2009c). *In vitro* studies also indicate that depending on the aminoglycoside resistance mechanisms present, Plazomicin may have activity against select isolates of *P. aeruginosa* and *A. baumannii* (Aggen et al., 2010; Landman et al., 2011). Susceptibility to plazomicin in the setting of resistance to other aminoglycosides appears to be dependent on the mechanism of aminoglycoside resistance (Livermore et al., 2011a).

NAB739 and NAB7061 are polymyxin derivatives that may be less nephrotoxic than commercially available polymyxins. In a small *in vitro* study, NAB739 displayed activity against nine carbapenemase-producing polymyxin-susceptible isolates of Enterobacteriaceae (Vaara et al., 2010). A contemporary evaluation of NAB739 demonstrated higher MICs compared to those of polymyxin B in a collection of polymyxin-susceptible and non-susceptible Enterobacteriaceae, *P. aeruginosa*, and *A. baumannii* (Vaara et al., 2012). NAB7061 when used in combination with rifampicin or clarithromycin demonstrated synergistic activity against seven strains of carbapenemase-producing Gram-negative bacilli including one polymyxin-resistant strain (Vaara et al., 2010). It remains unclear what role these agents will play in the setting the increasing burden of infections with carbapenemase-producing Enterobacteriaceae.

The activity of the siderophore monosulfactam, BAL30072, has been against non-fermenting carbapenemase-producing Gram-negative bacilli (Page et al., 2010). In one study, susceptibility to BAL30072 was noted in 73% of 200 isolates of carbapenemase-producing *A. baumannii*, the majority of which were of the same OXA-23 producing clone (Mushtaq et al., 2010a). In that same study, smaller percentages of susceptibility were noted in a selection of carbapenem-resistant *Burkholderia cepacia* and *P. aeruginosa* isolates. Recent evaluations of BAL30072 confirm that there may be a role for this agent in the treatment of resistant *A. baumannii* infections (Russo et al., 2011; Higgins et al., 2012b). BAL 30376 is a combination of a siderophore monobactam with clavulanic acid. In two studies, this combination demonstrated reasonable *in vitro* activity against CHDL, including OXA-48, and MBLs but not KPCs (Livermore et al., 2010; Page et al., 2011).

CONCLUDING REMARKS

In the last 5 years, we have witnessed the global spread of carbapenem resistance among Gram-negative organisms. The notion that multidrug resistance among these pathogens is limited to isolated outbreaks among the critically ill has met the ultimate challenge with NDM-1 (Kumarasamy et al., 2010). The conveniences of travel and medical tourism have introduced resistance mechanisms across states, countries, and even continents at an alarming rate (Rogers et al., 2011; van der Bij and Pitout, 2012). Rates of resistance in some countries may be underestimated due to the lack of organized reporting structures and limited resources. Long-term healthcare facilities are now recognized reservoirs for the continued propagation of MDR organisms (Urban et al., 2008; Aschbacher et al., 2010; Perez et al., 2010a; Ben-David et al., 2011; Prabaker et al., 2012; Viau et al., 2012).

Until the introduction of accurate, affordable, and readily accessible diagnostics and reliably effective antimicrobials a major focus remains containment and eradication of these organisms within the healthcare environment. Many cite a "bundle" type approach that includes administrative support, active surveillance, antimicrobial stewardship, and augmented infection control practices (Centers for Disease Control and Prevention, 2009; Schwaber et al., 2011; Snitkin et al., 2012). Just as with drug development (Tillotson, 2010), the future savings of investing in prevention is not as tangible as the immediate capital investment required to allot appropriate resources including advanced laboratory platforms, experienced laboratory personnel, dedicated nursing staff, and infection control personnel (Bilavsky et al., 2010). Expanding these efforts to non-acute healthcare settings is recommended to begin to stem the evolving pandemic of carbapenem resistance (Gupta et al., 2011).

The prudent use of antibiotics is essential in combating the continuing evolution of resistance (Marchaim et al., 2012). This may be even more crucial in areas where non-prescription antimicrobial use is common and continues to be unregulated. In an age where multidrug resistance is so widespread, even the appropriate use of broad-spectrum antibiotics has contributed to our current state.

Research funding and support for the description of resistance mechanisms, validation of current infection control practices, and antimicrobial development must be prioritized. Institutions supporting infection control, state of the art microbiology laboratories, and antimicrobial stewardship programs should receive recognition and incentives for their foresight. Despite these continuing challenges, considerable progress has been made to identify at-risk populations and to describe resistance determinants. Collaborative efforts (Kitchel et al., 2009a; Struelens et al., 2010; Canton et al., 2012) have led to a better understanding and awareness of the epidemiology and the contribution of antimicrobial use and the environment to the propagation of antimicrobial resistance. These joint efforts have proven crucial for the propagation of information about carbapenemases. Continuing to encourage these partnerships is imperative in the ongoing struggle against antimicrobial resistance and to prevent antimicrobials from essentially becoming obsolete.

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The multifaceted roles of antibiotics and antibiotic resistance in nature

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Antibiotics are chemotherapeutic agents, which have been a very powerful tool in the clinical management of bacterial diseases since the 1940s. However, benefits offered by these magic bullets have been substantially lost in subsequent days following the widespread emergence and dissemination of antibiotic-resistant strains. While it is obvious that excessive and imprudent use of antibiotics significantly contributes to the emergence of resistant strains, antibiotic resistance is also observed in natural bacteria of remote places unlikely to be impacted by human intervention. Both antibiotic biosynthetic genes and resistance-conferring genes have been known to evolve billions of years ago, long before clinical use of antibiotics. Hence it appears that antibiotics and antibiotics resistance determinants have some other roles in nature, which often elude our attention because of overemphasis on the therapeutic importance of antibiotics and the crisis imposed by the antibiotic resistance in pathogens. In the natural milieu, antibiotics are often found to be present in sub-inhibitory concentrations acting as signaling molecules supporting the process of quorum sensing and biofilm formation. They also play an important role in the production of virulence factors and influence host–parasite interactions (e.g., phagocytosis, adherence to the target cell, and so on). The evolutionary and ecological aspects of antibiotics and antibiotic resistance in the naturally occurring microbial community are little understood. Therefore, the actual role of antibiotics in nature warrants in-depth investigations. Studies on such an intriguing behavior of the microorganisms promise insight into the intricacies of the microbial physiology and are likely to provide some lead in controlling the emergence and subsequent dissemination of antibiotic resistance. This article highlights some of the recent findings on the role of antibiotics and the genes that confer resistance to antibiotics in nature.

Keywords: antibiotics, sub-inhibitory concentration, quorum sensing, virulence, stress response, antibiotic resistance, antibiotic paradox

INTRODUCTION

The term "antibiotics" was first coined by the American microbiologist Selman Waksman and his colleagues to describe chemical substances produced by microorganisms and having antagonistic effects on the growth of other microorganisms. It excluded synthetic antimicrobials (sulfur drugs) and biological products of non-microbial origin having antagonistic effects on bacteria. Though antibiotics were introduced into the clinical practice only in the middle of the last century, the use of microorganisms for the management of microbial infections in ancient Egypt, Greece, China, and some other places of the world is well-documented. The modern era of antibiotics started with the serendipitous discovery of penicillin from the culture filtrate of a fungus, *Penicillium notatum* by Alexander Fleming in 1928 (Fleming, 1929).

In the present scenario, antibiotics available in the market are either produced by microbial fermentation or are derived via semi-synthetic route using the existing antibiotic backbone structure. They are classified into different chemically defined groups. Antibiotics target bacterial physiology and biochemistry, causing microbial cell death or the cessation of growth. A significant number of these antibiotics affect cell walls or membranes (e.g., β-lactam and glycopeptides), while several others exert their antibacterial activity by targeting protein synthetic machinery via interaction with ribosomal subunits and these include antibiotics such as macrolides, chloramphenicol, tetracycline, linezolid, and aminoglycosides. Other "mechanistic" groups include molecules which interfere with the nucleic acid synthesis [e.g., fluoroquinolones (FQ) and rifampin], while some others exert their effects by interfering with the metabolic pathways (e.g., sulphonamides and folic acid analog) or by disruption of the bacterial membrane structure (e.g., polymyxins, daptomycin, and others).

A surge of discovery of several such antibacterial and antifungal antibiotics accompanied with a new generation of semi-synthetic drugs initially led to euphoria that any infectious disease could be successfully controlled using antibiotics. However, emergence and

propagation of bacterial strains, resistant to almost all the therapeutically useful antibiotics during the past few decades revealed the limitation of the wonder drugs. Though imprudent and excessive use of antibiotics is highlighted as a major causative factor behind the setback, it is evident by this time that antibiotic resistance does not call for exposure of the organisms to antibiotics. It is also found that genes involved in the biosynthesis of antibiotics and antibiotic resistance evolved thousands of years before antibiotics were introduced into the clinical practice. Hence both antibiotic and its resistance determinants have some other role in bacterial physiology.

ROLE OF ANTIBIOTICS IN NATURE

Antibiotics in the biosphere are produced by microorganisms as secondary metabolites at a concentration much lower than the therapeutic dose. Waksman was convinced that antibiotics play "no real part in modifying or influencing living processes that occur in nature" (Waksman, 1961) though there is evidence to the contrary (Gullberg et al., 2011). Antibiotics are produced in the late stages of microbial stationary growth phase, decoupled from the doubling time, implying that they are not indispensable for sustenance of life of the producer organism. However, it is also a fact that the production of an antibiotic is a multistep process that involves a number of genes. Hence it does not seem tenable that such a complex anabolic process has been sustained through evolution without having any obvious purpose to serve. Recent studies reveal that antibiotics do have some specific effects on the natural milieu of the microbes while they assume an entirely different role as antibacterial agents in the dose used in therapeutics. This dual role of antibiotics remains a riddle. In the natural habitat, the diverse microbial communities exist as a multi-cellular network. They constitute a huge reservoir of different metabolic activities and have to evolve continuously to encounter the constant environmental threat and different selection pressure. Survival is most often a major challenge because of the limiting nutrients. Under nutrient starvation, the organisms start secreting secondary metabolites, which are a diverse array of low molecular weight organic molecules, known as the parvome (Davies, 2009). The total number of parvome detected so far is at least an order of magnitude larger than the number of bacteria in the biosphere. Bacteria of the phylum Actinobacteria (Ventura et al., 2007), a huge taxonomic group of diverse genera with a high genomic guanine–cytosine (GC) content, produce millions of complex bioactive small molecules. The ecological role of the majority of these small molecules in nature remains largely unknown. Amongst these molecules, only a fraction has been identified to have antibiotic activity and has been extensively studied only from the therapeutic point of view whereas the significance of antibiotics in nature still remains a mystery. The prevailing confusion about its role in nature has been dealt by Julian Davis in an article titled: "are antibiotics naturally antibiotic?" (Davies, 2006).

ROLE OF ANTIBIOTICS IN INTERSPECIES COMPETITION

Antibiotics are critical to the producer organisms in the natural environment as they are needed both for survival and competitive advantage. Thus antibiotics are widely perceived as an arsenal of the producer microorganisms that they use against other naturally occurring cohabiting microorganisms and eliminate these competing bacteria for the purpose of "empire building" in the microbial community (Davies, 1990). The concept of the empire building role of antibiotics stemmed from the soil borne nature of the antibiotic-producing microorganisms. In certain instances like plant–microbe interactions, the antibiotic-producing organisms have indeed been found to secrete antibiotics to eliminate the competing bacteria in the vicinity. *Pseudomonas fluorescens* 2-79 (NRRL B-15132) colonizing the rhizosphere of wheat, secrete the antibiotic, phenazine which inhibits the growth of *Gaeumannomyces graminis* var. *tritici* thus suppressing a major root and crown disease of wheat and barley (Thomashow and Weller, 1988). Few other ecological examples of probable antibiotic function of these secondary metabolites in nature include the fungus-growing ants, which carry an antibiotic-producing actinomycete (*Pseudonocardia* sp.) on their cuticle specifically for biocontrol of the fungal garden parasite, *Escovopsis* sp. (Currie et al., 1999; Cafaro and Currie, 2005). Another example is the antibiotic-producing strain *Streptomyces diastatochromogenes* strain PonSSII controlling the growth of *Streptomyces scabies* strain RB4, the causative agent of potato scab (Neeno-Eckwall et al., 2001).

ROLE OF ANTIBIOTICS AS SIGNALING MOLECULES

During primordial development, the cells instead of antagonizing each other remained in a communal harmony to share an evolutionary advantage and the symbiotic coexistence of microbes in biofilms, in lichens and in a metabolically quiescent state is well-documented (Davies, 2006). Chemical communication, an essential feature in such mixed populations, helps the microorganisms coordinate with each other in an orchestrated fashion. Probably the small molecule antibiotics act as a liaison in the communication between the microbes. It has been postulated that antibiotics in the early stages of biochemical evolution had some functions mediated by their interaction with primitive macromolecule receptors. Later on, many of these low molecular weight interacting partners were employed to antagonize the original receptor sites in macromolecular structure thus imparting the antibiosis property. Such a switchover in the role of the primordial effector molecule has been hypothesized in a schematic biochemical pathway by J. Davies (Davies, 1990). During the past seven decades, the scientists were mostly preoccupied with studying the antibiotic property of these small molecules because of its overwhelming success in pharmacotherapy. Only during the recent past antibiotics were implicated in several other physiological phenomena ranging from control of transcription and translation to growth of the producer organism in the microbial communities. From a broader perspective of evolutionary and ecological point of view, antibiotics, apart from having a growth inhibitory activity, appear to have a role in intra- and inter-domain communication in various ecosystems. Under normal physiological condition, microorganisms in their marine and soil habitats produce antibiotics at sub-minimum inhibitory concentration (sub-MIC). Despite having evidence that resistant bacteria representing a low fraction in a sample were enriched at sub-inhibitory concentrations of three clinically useful antibiotics (Gullberg et al., 2011), antibiotics are believed not to display any antibiotic activity

at concentrations lower than 1/100 of the minimum inhibitory concentrations (Davies, 2006). Instead, many antibiotics are known to possess biological activities other than the inhibitory role at such a low concentration and have major effects on global transcription pattern. Antibiotics such as erythromycin (an inhibitor of translation) and rifampicin (an inhibitor of transcription), at low concentrations, could modulate global bacterial transcription pattern in a random promoter library construct of *Salmonella typhimurium* having *lux as the* reporter gene. In response to the low concentration of different antibiotics, a variety of promoters were activated, including those involved in virulence, metabolic, and adaptive functions. However, transcription was markedly reduced in antibiotic-resistant hosts but the mutants defective in stress responses such as *rec* and *lex* (SOS), *dnaJ* and Δ*dnaKJ* (heat shock response), and Δ*relA* Δ*spoT* (universal stress response) could not prevent antibiotic-induced modulation of transcription pattern at sub-inhibitory concentration (Goh et al., 2002). Antibiotics having different binding sites on the ribosome (chloramphenicol, aminoglycosides, macrolides, and tetracycline), or intruding into the process of cell-wall synthesis (some β-lactams and fosfomycin) were reported to alter the transcription pattern under different circumstances. So it has been postulated that the transcription machinery must be capable of sensing these subtle variations in the conformation or stoichiometry of small molecule antibiotics to respond to specific up- or downregulation.

These small molecules have a significant role in the dynamics of bacterial communities in nature, thus contributing to both competitive and interactive responses. Inhibition occurs when high concentrations are attained, transcriptional changes occur at low concentrations (Davies et al., 2006). This dose-dependent dual role, defined as hormesis, is not exclusively associated with antibiotics but also found in different other biomolecules (Davies et al., 2006; Calabrese, 2009). The dual role of antibiotics has been confirmed by the detection of a variety of bioactivities of several antibiotics. Some β-lactam antibiotics were shown to stimulate the glutamate transporter-1 (GLT1) both in cell culture and in animal models (Rothstein et al., 2005). Thiostrepton, a potent protein synthesis inhibitor which binds to 23S RNA, was found to act as a transcription inducer at a concentration lower than the inhibitory concentration (Murakami et al., 1989). Lincomycin, which terminates the peptide bond elongation, could stabilize certain mRNAs like the *bla* mRNA (Matsushita et al., 1989). Puromycin, in spite of having a chain termination activity in polypeptide synthesis, can affect nucleic acid synthesis, mediated by its aminonucleoside moiety (Yarmolinsky and Haba, 1959). Edeine, apart from its inhibitory role in protein synthesis, has specific effects on nucleic acid synthesis (Kurylo-Borowska and Szer, 1972). Many of the so-called antibiotics have been shown to have sex pheromone activity and found to stimulate bacterial conjugation by several orders of magnitude (Evans and Dyke, 1988). Sub-MICs of antibiotics are thus known to induce extensive transcriptional changes in bacteria (Tsui et al., 2004; Yim et al., 2006a). In the majority of cases the underlying mechanisms of the different ecological role of the antibiotics at sub-inhibitory concentration still remain to be elucidated.

A second level of signaling via antibiotics has also been documented in different organisms. Cationic peptides, which have a broad range of antimicrobial activities against most Gram-negative and Gram-positive bacteria, fungi, enveloped viruses, and eukaryotic parasites, also have a broad spectrum of pro-inflammatory and anti-inflammatory effects on the host immune system. Apart from their antimicrobial activity, these molecules also act as transcription modulators. Cecropin, an insect-derived cationic peptide, was shown to alter the transcription pattern of 26 genes when added to *Escherichia coli* (Hong et al., 2003). Some cationic peptides of microbial- and mammalian origin (polymyxins B and E, cattle indolicidin and human LL-37) were found to activate the two component *pmrA–pmrB* regulatory operon, which regulates the resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa* (McPhee et al., 2003). Inhibitory effect of several antibiotics on sporulation of bacteria is well-known (Ochi and Freese, 1983; Babakhani et al., 2012).

ROLE OF ANTIBIOTICS IN VIRULENCE

Some antibiotics, at concentrations below the MIC, are able to modulate the expression of some genes associated with virulence. Up- and downregulation of the transcription of virulence and motility genes were induced by the RNA polymerase inhibitor, rifampicin (Yim et al., 2006b). Sub-inhibitory concentration of several other antibiotics such as metronidazole, vancomycin, clindamycin, and linezolid could induce the early transcription of *tcdA* (*toxin A*) and *tcdB* (*toxin B*), the major virulence factors-encoding genes of *Clostridium difficile*, a nosocomial pathogen responsible for diarrhea, during exponential phase growth of the organism (Gerber et al., 2008). Imipenems, at sub-inhibitory concentration, were shown to induce β-lactamase and production of alginate during biofilm formation in *Pseudomonas aeruginosa*. Sub-inhibitory concentration of an aminoglycoside antibiotic tobramycin induced biofilm formation in *Pseudomonas aeruginosa* and *Escherichia coli* (Hoffman et al., 2005). On the other hand, sub-inhibitory level of the macrolide antibiotic azithromycin was shown to elicit the exactly opposite phenomenon like suppression of the alginate overproduction and biofilm formation in *Pseudomonas aeruginosa* (Ichimiya et al., 1996). These contradictory observations suggest that the molecular targets of different antibiotics for the execution of their inhibitory effects could be similar but they might differ extensively while functioning as signaling molecules (Aminov, 2009). This postulation is substantiated by the fact that tobramycin at sub-inhibitory concentrations does not bind the ribosome (the usual target for its role as an antibiotic), rather it targets the aminoglycoside response regulator gene (arr) which is responsible for the induction of biofilm-specific aminoglycoside resistance. The gene is also predicted to encode an inner-membrane phosphodiesterase acting on the substrate, cyclic di-guanosine monophosphate (c-di-GMP), a bacterial second messenger that regulates cell surface adhesiveness (Hoffman et al., 2005).

The influence of sub-inhibitory doses of several antibiotics on the expression of the gene encoding the *Staphylococcus aureus* alpha-toxin (*hla*), a major virulence factor of the pathogen, revealed that glycopeptide antibiotics had no effect whereas β-lactams induced a strong expression of *hla*. On the other hand, the macrolide erythromycin and several aminoglycosides reduced, while FQs slightly stimulated *hla* expression and an almost

complete inhibition of alpha-toxin expression was obtained using clindamycin. Furthermore, methicillin enhanced Hla production of both MSSA (methicillin-sensitive *Staphylococcus aureus*) and MRSA (methicillin-resistant *Staphylococcus aureus*; Ohlsen et al., 1998). The sub-inhibitory concentration of ceftazidime (β-lactam) and ciprofloxacin (FQ) appeared to be effective in decreasing the expression of a range of quorum sensing (QS)-regulated virulence factors (Skindersoe et al., 2008).

Genomic rearrangements in pathogenic strains of *Escherichia coli* and *Shigella* following exposure to sub-inhibitory concentrations of kanamycin and ampicillin was demonstrated (Pedro et al., 2011). Some of the rearrangements may engender genotypes with increased virulence. SOS response, induced by ciprofloxacin, was found to stimulate transfer of pathogenicity Island in *Staphylococcus aureus* (Ubeda et al., 2005). Thus antibiotic used for the treatment of clinical infections may lead to the dissemination of virulence in some cases.

ROLE OF ANTIBIOTICS IN HOST–PARASITE INTERACTION

Antibiotics have also been found to modulate host immune cell response, mostly mediated by cytokines. There are ample evidences of sub-inhibitory concentrations of antibiotics interference with processes of host–parasite interactions such as phagocytosis, adherence, and virulence. Antibiotics promote phagocytosis by altering the surface properties of pathogens. Many clinical isolates of *Enterococcus faecium* are resistant to neutrophil-mediated phagocytosis but exposure of vancomycin-susceptible *Enterococcus faecium* to quinupristin/dalfopristin at concentrations both at either sub-inhibitory or suprainhibitory concentrations promoted susceptibility to subsequent PMN (polymorphonuclear leucocytes)-mediated phagocytosis. Vancomycin-resistant strains on the contrary exhibited very little change in their binding toward PMNs after antibiotic pretreatment (Herrera-Insúa et al., 1997). Use of amoxicillin for the treatment of *Haemophilus influenzae* in acute otitis media caused upregulation in the expression of cytokines, interleukin (IL)-6, tumor necrosis factor-α, and IL-10 (Melhus, 2001).

ROLE OF ANTIBIOTICS IN SOS AND DNA REPAIR GENE EXPRESSION

The SOS response, a regulatory network present in most bacteria is induced in response to DNA damage and has been shown to promote stress-induced mutagenesis, virulence, and dissemination of antibiotic resistance specifically by horizontal gene transfer (HGT). Beaber et al. (2004) showed that ciprofloxacin induced transfer of the *Vibrio cholerae* SXT integrative conjugative element (ICE) was controlled by SOS through RecA-dependent cleavage of the SXT encoded repressor SetR. SXT is a 100-kb ICE derived from *V. cholerae* that encodes genes for the resistance toward chloramphenicol, sulfamethoxazole, trimethoprim, and streptomycin (Beaber et al., 2004). Even the induction of the CTX prophage which encodes the cholera toxin is regulated by the SOS repressor LexA in conjunction with the phage repressor rstR which is also regulated by LexA (Quinones et al., 2005). In *V. cholerae*, the recombination-mediated excision of integron cassette was induced by SOS in response to different antibiotics (Guerin et al., 2009). On the other hand, SOS-independent recombination between divergent sequences either by RecBCD or RecFOR pathways was demonstrated in *Escherichia coli* by quinolones (DNA-damaging antibiotics) induced emergence of drug resistance (López et al., 2007).

Fluoroquinolones at sub-MIC have been shown to upregulate both the SOS and the methyl mismatch repair (MMR) pathway genes in *Staphylococcus aureus* with different SOS response and DNA repair-associated promoter-*lux* gene fusion construct (Mesak et al., 2008). Upregulation of several genes involved in the SOS response such as *umuD*, *lexA*, *sbmC*, and *dinP* was demonstrated in *Salmonella* using sub-inhibitory concentrations of quinolones. In addition, transcriptional regulators, genes putatively associated with membrane integrity (*spr*), virulence (*sicA*), and metabolism (*plsB*) were also affected (Yim et al., 2011). On the contrary, it has also been shown that exposure of *Streptococcus pneumoniae* to quinolones, aminoglycosides, and penicillin at sub-inhibitory concentrations may result in increased mutability, without the apparent involvement of SOS-response components (Henderson-Begg et al., 2006; Cortes et al., 2008).

SOS response also enhances frequency of mutations. Hence antibiotics which induce SOS response, contribute to the increase in the frequency of resistant strains. Exposure to ciprofloxacin was found to increase in the frequency of rifampicin-resistant mutants of *Streptococcus pneumoniae* almost by fivefold (Henderson-Begg et al., 2006). Carbapenem resistance in *Pseudomonas aeruginosa* was also found to be enhanced in presence of the same antibiotic almost in the same order (Tanimoto et al., 2008).

Thus low antibiotic concentration regulates the transcription of different genes in target bacteria while increasing higher concentration elicits a stress response and even higher concentration is lethal. Therefore, it is apparent that these small molecule at a sub-inhibitory concentration participate in a new form of signaling network by binding to different cytoplasmic macromolecular receptors such as ribosomes, and those involved in DNA replication, RNA transcription machinery, and cell-wall biosynthesis (Ryan and Dow, 2008). These receptors were also identified earlier as the inhibitory targets for the bioactive molecules at higher concentration. The cellular targets for the lethal and sub-inhibitory concentration sometimes also differ thus implying that antibiotics are multi-ligand-specific sensor molecules targeting different ligands in a concentration-dependent manner (Hoffman et al., 2005).

ANTIBIOTICS AND QUORUM SENSING

Quorum sensing is a mode of bacterial communication in response to release of chemical messenger which regulate bacterial gene expression (Bassler, 1999). Secondary metabolites are activated during slow growth in the stationary phase and trigger the transition from primary to secondary metabolism (Bibb, 2005). This transition is a complex process and involves many signals, including those by small signaling molecules called γ-butyrolactones (also known as autoregulatory factor or A-factor). These signaling molecules, found mainly in *Streptomyces* species, are considered "bacterial hormones" or the QS factor. The QS signaling network initiates a set of metabolic changes leading to a number of events including morphological differentiation and synthesis of secondary metabolites such as antibiotics. The γ-butyrolactones constitute the major group of QS molecules in

bacteria. The first γ-butyrolactone, A-factor, was identified in *Streptomyces griseus* in 1967 (Hsiao et al., 2009).

Biosynthesis of γ-butyrolactones is not well understood, but seems to be mediated by γ-butyrolactone synthase AfsA. The A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactones), at a certain critical concentration, binds to cytoplasmic receptor protein, a dimer of ArpA, which primarily acts as repressors by binding to the promoter region of adpA (A-factor-responsive transcriptional activator, a key pleiotropic regulator). The binding of the A factor dissociates ArpA from the promoter, thus facilitating the transcription and translation of adpA. Subsequently, AdpA binds an upstream activation sequence to initiate the transcription of strR (a pathway-specific regulatory gene responsible for transcription of other streptomycin biosynthetic genes cluster). strR is a pathway-specific regulator which acts as a transcriptional activator and induces transcription of most of the streptomycin biosynthetic genes by binding multiple sites in the gene cluster (Retzlaff and Distler, 1995). Thus onset of streptomycin biosynthesis is initiated by the QS factor, γ-butyrolactones. The gene *aph*D, encoding a major streptomycin resistance determinant streptomycin-6-phosphotransferase, is also under the control of the *adp*A-dependent promoter. The binding to γ-butyrolactones thus induces expression of the target genes (transcription factors) many of which are involved in regulation of specific antibiotic biosynthesis clusters. Onset of streptomycin biosynthesis is initiated by the QS factor, γ-butyrolactones (Retzlaff and Distler, 1995). The expression of the gene *aph*D, encoding a major streptomycin resistance determinant streptomycin-6-phosphotransferase, is also a QS-mediated event. Each receptor protein is highly specific for its cognate γ-butyrolactone. In *Erwinia carotovora* carbapenem biosynthesis is regulated by a classical autoinducer *N*-(3-oxohexanoyl)-L-homoserine. In another cephamycin C-producing bacterium, *Streptomyces clavuligerus*, antibiotic synthesis is regulated at the primary regulatory level by γ-butyrolactone (Liras et al., 2008).

Some 10–11 probable γ-butyrolactone synthates, all from the *Streptomyces* genus were found while 37–42 putative γ-butyrolactone receptors were found in the genome of different other bacteria apart from *Streptomyces*. Thus, it is believed that many of the cellular activities including the antibiotic production is under the control of a well-orchestrated QS signaling network mediated by a small population of γ-butyrolactone producers acting as a signaling factor for a diverse population of signal recipient (Aminov, 2009).

Structural similarity between antibiotic and intracellular signaling molecule has also been evidenced in several cases. The quinolones produced by *Pseudomonas aeruginosa* have a wide range of activities starting from antibiotics to autoinducers. 2-Heptyl-3-hydroxy-4-quinolone (pseudomonas quinolone signal; PQS), belonging to the family of 2-alkyl-4-quinolones (AQs), was previously described for their antimicrobial activities. Later on, it was found that PQS is integrated within an intricate QS circuit and plays an important role in *Pseudomonas aeruginosa* pathogenesis by regulating the production of diverse virulence factors including elastase, pyocyanin, and LecA lectin in addition to affecting biofilm formation (Dubern and Diggle, 2008; Heeb et al., 2011). Several other autoinducers like *N*-(3-oxododecanoyl) homoserine lactone and its tetrameric acid degradation product in *Pseudomonas aeruginosa* have antibacterial properties against Gram-positive bacteria (Kaufmann et al., 2005). *N*-(3-oxododecanoyl) homoserine lactone and other 3-oxo series homoserine lactones with 8, 10, and 14 length carbon chains were shown to inhibit growth of the Gram-positive *Staphylococcus aureus* (Qazi et al., 2006).

ANTIBIOTIC RESISTANCE, THE TIP OF THE ICEBERG

In the late 1960s, the unprecedented successes of early antibiotic therapies led US Surgeon General William H. Stewart to make the famous declaration: "it is time to close the book on infectious diseases and declare the war against pestilence won" (Spellberg et al., 2008). The euphoria did not last long and the magic bullets started losing the efficacy because of the steady emergence of antibiotic-resistant pathogens simultaneously with their widespread use. Following the success of penicillin in controlling bacterial infection among the soldiers during the Second World War, emergence of penicillin-resistant strain was evidenced in the 1940s. By 1960, it assumed the shape of a pandemic problem. New β-lactam antibiotics were introduced into the clinical practice to restrain the problem. Simultaneously bacterial strains resistant to them came into being, a phenomenon dubbed as β-lactamase cycle. The first case of MRSA was identified in UK in 1961 (Johnson, 2011) and the first report on MRSA in the United States came in 1968. Now it is prevalent all over the world. MRSA is actually resistant to the entire class of penicillin-like antibiotics called β-lactams. Presence of an enzyme called New Delhi metallo-β-lactamase (NDM-1) in some Gram-negative bacteria (notably *Escherichia coli* and *Klebsiella pneumoniae*) makes them resistant to virtually all β-lactams including carbapenems, which are most often considered the last line of defense against multidrug-resistant pathogens (Kumarasamy et al., 2010). Isolation of bacterial strains producing extended spectrum β-lactamase (ESBL) resistant to third generation of cephalosporins and monobactams is also reported from time to time (Kurihara et al., 2013).

The glycopeptide antibiotic vancomycin was introduced in clinical practice in 1958 for the treatment of methicillin resistance in both *Staphylococcus aureus* and coagulase-negative staphylococci. Vancomycin resistance was so difficult to induce that it was believed to be very unlikely to occur in a clinical setting. Until the late 1980s, the glycopeptide antibiotic vancomycin was considered the drug of last resort for treatment of diseases caused by Gram-positive bacteria such as *Enterococcus faecalis*, MRSA, *Streptococcus pneumoniae*, and *Clostridium difficile* (Cunha, 1995). However, vancomycin resistance was first reported in coagulase-negative staphylococci in 1979 and 1983 (Srinivasan et al., 2002). At present vancomycin-resistant enterococci (VRE) pose a major challenge in therapeutics. Multidrug-resistant tuberculosis (caused by *Mycobacterium tuberculosis*) has assumed an epidemic proportion in some parts of the world. Multidrug-resistant strains of *M. tuberculosis*, now known to be present in 50 countries, heighten the threat posed by untreatable and fatal human tuberculosis.

Because of the rapid dissemination of antibiotic resistance in pathogens, many of the antibiotics, which were highly effective earlier, became obsolete during the past few decades. The efficacy of antibiotic treatment is on the wane as a result of the emergence and

dissemination of antibiotic resistance amongst the pathogens. This warrants the discovery of newer and more promising antibiotics with long-term efficacy against various life threatening infections. However, the steady discovery of antibiotics witnessed during 1940–1980 could not be sustained. In the last two decades, only one new antibiotic class viz the oxazolidinone was introduced in the year 1990 and all other entrants were just the variation of the existing one (Raghunath, 2008). Eventually, clinical management of antibiotic-resistant superbugs is assuming the shape of a gruesome problem because of the depleting antibiotic reserve (Norrby et al., 2005). The widespread apprehension that we might be heading to a situation similar to the pre-antibiotic era cannot be dismissed as a far-stretched imagination.

Imprudent and excessive use of antibiotics is highlighted as a major causative factor behind the setback. The idea dubbed as Antibiotic Paradox by Levy (1992) is corroborated by numerous evidences. In 1967, the first penicillin-resistant *Streptococcus pneumonia* was observed in Australia, and after 7 years another case of penicillin-resistant *Streptococcus pneumonia* was reported from the U.S. in a patient with pneumococcal meningitis (Doern et al., 2001). In 1980, 3–5% of *Streptococcus pneumonia* was estimated to be penicillin-resistant and by 1998, 34% of the *Streptococcus pneumonia* sampled was resistant to penicillin (Doern et al., 2001). Antibiotic resistance in other organisms reflects the same trend as observed in *Streptococcus pneumonia* against penicillin. Tetracycline resistance by normal human intestinal flora exploded from 2% in the 1950s to 80% in the 1990s (Shoemaker et al., 2001). Kanamycin, an antibiotic used in the 1950s, has become clinically obsolete as a result of the prevalence of kanamycin-resistant bacteria (e.g., *M. tuberculosis*, *Campylobacter jejuni*).

On the other hand, it is also a fact that antibiotic resistance is observed even in bacteria isolated from totally uninhabited and thinly populated places, where they are unlikely or least likely to come in contact with the antibiotics (Chattopadhyay and Grossart, 2010). It is evident that antibiotic resistance is an outcome of evolution and pre-exposure of microorganisms to antibiotics is not a pre-requisite for emergence of resistance. In a systematic analysis, the conjugative plasmids occurring in several bacteria belonging to Enterobacteriaceae obtained from Murray collection were found to belong to the same incompatibility group as those present in contemporary Enterobacteriaceae (Datta and Hughes, 1983). It was shown that emergence and spread of conjugative plasmids, which are known to be a major vehicle for transmission of resistance, were not fostered by the widespread use of antibiotics, rather these plasmids evolved by inclusion of resistance-conferring genes in pre-existing plasmids (Datta and Hughes, 1983; Hughes and Datta, 1983).

Despite intensive investigations continuing for the last few decades in a global scale, no significant achievement has been made so far in the prevention and control of resistance development. A recent database reveals the existence of more than 20,000 potential resistance genes of nearly 400 different types in the available bacterial genome sequences. The fact that the number of functional resistance determinants in pathogens is much smaller (Liu and Pop, 2009) provides no relief since the new generation of therapeutically useful antibiotics are reaching the market only in trickle.

The World Health Organization (WHO) has long recognized anti microbial resistance (AMR) as a growing global health threat, and the World Health Assembly, through several resolutions over two decades, has called upon Member States and the international community to take measures to curtail the emergence and spread of AMR.

EMERGENCE OF RESISTANCE PHENOTYPE

Antibiotic resistance is known to occur both in the pre-antibiotic and antibiotic-era. The pre-antibiotic era constitutes the time before the introduction of sulphonamides in 1930. It is well established that resistance phenotype and the antibiotics in the pre-antibiotic era coexisted in the natural environment without facilitating the process of selection of the deadly resistant pathogens.

The antibiotic era started following its discovery of antibiotics and their use in different spheres of life. The use of high concentration of lethal dose of antibiotics as a consequence of human activity led to a major change in innate functional role to give rise to the emergence of antibiotic-resistant pathogens within a short span of time. Antibiotic resistance genes, which were once involved in other cellular functions before human intervention, have been subsequently selected for the resistance phenotype with increased use of antibiotics. They have been mobilized from the environmental genomic reservoirs, with the rapid dissemination into taxonomically divergent commensal and pathogenic bacteria. Metagenomic approach in conjunction with a pipeline for the *de novo* assembly of short-read sequence data from functional selections (termed PARFuMS) has shown the evidence of lateral transmission of five different antibiotic resistance genes along with different non-coding region as well as multiple mobilization sequences from environmental reservoir of soil bacteria to the clinical pathogens (Forsberg et al., 2012). Thus HGT or lateral gene transfer, which contributes significantly to the evolution, maintenance, and transmission of virulence in pathogenic bacteria, also plays a pivotal role in dissemination of antibiotic resistance conferring genes from the environment in clinical settings (Colomer-Lluch et al., 2011).

Epigenetic inheritance-based evolution of antibiotic resistance genes has been reported in an isogenic population of *Escherichia coli*, exposed to gradually increasing concentration of different antibiotics such as ampicillin, nalidixic acid, and tetracycline. The high frequency of survival on low antibiotic concentration could not be accounted for by the occurrence of random spontaneous mutation. Instead it suggested that the antibiotic resistance genes were acquired by epigenetic inheritance. High reversion of this resistance phenotype further proved that it was indeed a case of epigenetic inheritance which does not impart a stable phenotype due to maintenance of certain chromatin configuration or DNA methylation state (Adam et al., 2008).

There are ample evidences to suggest that anthropogenic factors (use of antibiotics by human both for therapeutic and non-therapeutic purposes and disposal of the unused antibiotic formulations) inflict significant changes on the natural flora of bacteria. But the actual concentrations of antibiotics (and other pharmaceuticals) that bacteria in the natural population is exposed to, remain unknown in most of the cases (Kümmerer, 2003, 2004).

Its role in the emergence of antibiotic resistance still remains highly controversial (Bhullar et al., 2012).

In therapeutics, the pathogens are challenged with an overwhelmingly high concentration of a single or few antibiotics whereas the soil borne microbes exist in a complex microenvironment and encounter a number of simultaneously occurring stress factors in a network of multiple interactions. Probably, these interactions have a nullifying effect on each other thus favoring the evolutionary selection of antibiotic sensitivity over resistance (Chait et al., 2011). Therefore, the dynamics of antibiotic resistance in clinical settings is believed to be inflicted by anthropocentric factors and is quite different from naturally occurring resistance. Simulation of these natural niches may reveal the dynamics by which antibiotic resistance is disseminated across the microbial population.

IMPLICATION OF SUBLETHAL CONCENTRATIONS OF ANTIBIOTICS IN DISSEMINATION OF RESISTANCE

In nature, sub-inhibitory concentrations of antibiotics are probably encountered by bacteria more frequently than inhibitory concentrations. Most often bacteria are exposed to antibiotics at a sublethal concentration in certain clinical situations such as incomplete treatment of an infection, patient non-compliance, and reduced or limited drug accessibility to certain tissues (e.g., bone or cerebrospinal fluid; Bryskier, 2005). In the colon of a person taking antibiotics, sub-inhibitory concentrations would be experienced by colonic bacteria during the initial phase of treatment and at the end of treatment. Thus host tissues are exposed to a range of drug concentration starting from higher to a sub-inhibitory concentration. Therefore, micro-niches within the host, such as epidermis, lungs, and joints, may attain significantly lower drug concentrations than the plasma (Rybak, 2006). Outside clinical settings, sub-inhibitory condition is established because of the use of manure from livestock whose feed is supplemented with antibiotics and in consequence multiple drugs in a very low level find their way both to the soil and aqueous environment.

Due to such imprudent use of antibiotics, bacteria are quite likely to experience sublethal levels of antibiotics which have high implications in the spread of multidrug resistance. There is evidence that low level of antibiotics gives rise to mutagenesis in a wider range of antibacterial resistance genes and drug efflux systems resulting in multidrug resistance (Girgis et al., 2009). Sub-inhibitory concentrations of β-lactams were found to enhance the transfer of tetracycline resistance plasmids in *Staphylococcus aureus* by up to 1,000-fold (Barr et al., 1986). Ampicillin treatment of *Escherichia coli* was associated with the formation of norfloxacin-resistant isolates with mutations in *gyr*A, *gyr*B, or the *acr*AB promoter (PacrAB) and kanamycin-resistant isolates with mutations in *rpsL* or *arcA* (A and B). It has been hypothesized that low level of bactericidal antibiotics give rise to reactive oxygen species (ROS) which leads to DNA damage-induced mutations thus facilitating the emergence of multidrug-resistant bacteria (Kohanski et al., 2010). Induction of prophage in animal feed by antibiotics was evidenced. It may contribute to dissemination of antibiotic resistance (Allen et al., 2011). Induction of shigella toxin-encoding bacteriophage by ciprofloxacin and enhanced Shiga toxin (Stx) production from *Escherichia coli* O157:H7 was demonstrated *in vitro* and *in vivo*. Hence antibiotic-induction of phage may also contribute to increased virulence (Zhang et al., 2000).

In many cases, exposure to a low-dose antibiotic is associated with mobile element-mediated dissemination of antibiotic resistance genes through HGT which is known to be enhanced by sub-MIC concentrations of tetracycline (Celli and Trieu-Cuot, 1998). Tetracycline at a sub-inhibitory concentration in the mating medium substantially enhanced Tn916 mediated conjugal transfer to the recipient *Bacillus thuringiensis* subsp. *israelensis* (Showsh and Andrews, 1992). Spread of antibiotic resistance genes between human colonic *Bacteroides* spp. is mediated by self-transmissible elements known as conjugative transposons (CTns). The exposure of donor *Bacteroides* cells to low concentration of tetracycline appeared to be a pre-requisite for the excision of the CTnDOT family of CTns from the chromosome and conjugal transfer of the excised elements. Virtually no transfer occurs without the tetracycline induction of donor cells (Stevens et al., 1993; Whittle et al., 2002). Low concentrations of tetracycline might increase the likelihood of HGT of integrated mobile elements, such as CTnDOT and NBU1. At sub-inhibitory tetracycline concentration, the excision is not associated with growth phase (Song et al., 2005). Mobilization of coresident non-conjugative plasmids (from *Bacteroides* strain to *Bacteroides* strain or *Escherichia coli*) by chromosomally encoded tetracycline conjugal elements (Valentine et al., 1988) was enhanced by 20–10,000-folds when the donor was pre-grown in a sub-inhibitory concentration of tetracycline (1 μg/ml). The similar stimulatory effect of tetracycline on conjugation transfer was also demonstrated for the conjugative transposon Tn925 (Torres et al., 1991).

These *in vitro* observations were validated *in vivo* in gnotobiotic mice where tetracycline at a sublethal concentration in drinking water could induce the frequency of conjugative transfer of the transposon Tn1545 from *Enterococcus faecalis* to *Listeria monocytogenes* in the digestive tract and there was an approximately 10-fold increase in the transposition (Doucet-Populaire et al., 1991). In gnotobiotic rats, selection for the resistant phenotype was the major factor causing higher numbers of Tn916 transconjugants in the presence of tetracycline (Bahl et al., 2004). Therefore, the enhancement of conjugal transfer of antibiotic resistance-carrying transposons in the presence of sub-inhibitory concentration of antibiotics is not only an *in vitro* phenomenon but also takes place in the human intestinal microbiome of animal models.

Adverse effect of some antibiotics on bacterial motility was reported earlier (Molinari et al., 1992; Kawamura-Sato et al., 2000). However, sub-inhibitory concentration of tobramycin was observed to increase motility of *Pseudomonas aeruginosa* and enhance the expression of virulence determinants of the organism (Linares et al., 2006).

ANTIBIOTIC RESISTANCE AND ITS ROLE IN NATURE

Antibiotic resistance can be defined in different ways: from the microbiological point of view, the resistance is defined as a phenotype which makes the microorganism less susceptible than other members of the same species irrespective of any level of resistance. On the contrary, when the resistance reaches a certain critical level so as to interfere with the pharmacotherapy of a clinical problem

caused by the bacterium, it is called clinical resistance (Cantón and Morosini, 2011).

In the present scenario, acquired clinical resistance of pathogens is a major concern in the healthcare management which results from genetic changes involving mutation or HGT. Besides acquired resistance dealt with in this article, some other types of antibiotic resistances are also observed in bacteria. For example in some cases tolerance of a bacterium to a certain antibiotic is dependent on its metabolic state. It exhibits susceptibility to the antibiotic in growing state but become resistant to the same antibiotic in stationary phase. The micro-environments in a biofilm also contribute to the differential susceptibility of bacteria embedded in different parts of the polysaccharide matrix. Swarming, a phenomenon induced in some bacteria in response to nitrogen limitation, confers resistance to some antibiotics (Martinez and Rojo, 2011). Resistance to a variety of antibiotics was observed in swarming cells of some bacteria. This observation together with the resistance observed in biofilm clearly indicates that antibiotic resistance is a manifestation of social behavior of bacteria (Lai et al., 2009). Accumulation of the alarmone (p) ppGpp was also reported to modulate antibiotic resistance. Involvement of this alarmone in persistence (resistance occurring in a small fraction of naturally occurring bacteria) is postulated (Jayaraman, 2009). This type of resistance, known as phenotypic resistance, significantly contributes to some problems in clinical management of some bacterial infections.

The most ancient resistance also known as intrinsic resistance (Sheldon, 2005) is species-specific and results from impermeability of the cells to the antibiotic or lack of target of an antibiotic to a certain organism. However, besides these passive factors, intrinsic resistance is also caused by some antibiotic-detoxifying determinants encoding chromosomally encoded β-lactamases, efflux pumps or target-protecting proteins. This antibiotic resistance is widespread in nature and this huge intrinsic resistome of bacteria consists of genes of varied phylogenetic origin This intrinsic resistome has a high degree of non-specificity and many of the proteins encoded by these genes are involved in the basic physiological functions in the natural ecosystem as demonstrated in *Pseudomonas aeruginosa*, the novel nosocomial Gram-negative pathogen of environmental origin (Fajardo et al., 2008). The presence of antibiotics acts as a switch in physiological function to the antibiotic-resistance phenotype. These resistance genes persist in bacteria even in antibiotic-free environment thus further substantiating their alternative role in bacterial physiology. Tet(L) and Tet(K), the specific tetracycline c-resistance determinants are known to catalyze Na^+ and K^+ exchange for protons (Krulwich et al., 2001). Tet(L), a tetracycline-efflux transporter, and MdfA, a multidrug-efflux transporter, both responsible for alkali tolerance offer a striking example in support of this hypothesis (Krulwich et al., 2005). In *M. tuberculosis* the P55 efflux pump plays an important role in oxidative stress response and *in vitro* cell growth in addition to contributing to its intrinsic resistance phenotype (Ramón-García et al., 2009).

It is obvious that bacteria evolve with antibiotic resistance determinants get selective advantage over their antibiotic-sensitive counterparts in presence of antibiotics. However, some of the multidrug resistance determinants confer resistance not only to antibiotics but also to a number of structurally unrelated compounds viz, ethidium bromide, quaternary ammonium compounds, the DNA-intercalating mutagen acridine, the anionic detergent sodium dodecyl sulfate and uncouplers. Hence they appear to have a greater role in bacterial physiology. They also confer resistance to some chemical substances produced by the host, e.g., bile acids. It is speculated that antibiotic resistance is an offshoot of some hitherto undefined physiological roles played by the determinants in these bacteria (Martinez and Rojo, 2011).

Most antibiotic-producing strains carry genes encoding resistance to the antibiotics that they produce (Hopwood, 2007; Tahlan et al., 2007) and these genes are usually found in the same gene cluster as the antibiotic biosynthesis pathway genes (Benveniste and Davies, 1973; Martin and Liras, 1989). The proximity of the antibiotic resistance gene with the antibiotic biosynthetic gene as evidenced in *Streptomyces coelicolor* (where the genes encoding export proteins are embedded in the actinorhodin biosynthesis pathway) led to the postulation that these resistance determinants could have some role in the biosynthetic pathway of antibiotics (Allen et al., 2010). Antibiotic resistance genes are frequently present even in non-producer strains (Yamashita et al., 1985). In consequence, bacteria have evolved with a diverse pool of genes (the "resistome") that protect them against the therapeutic dose of antibiotics. Gene orthologous to these have been identified on mobile genetic elements in resistant pathogens in clinical settings. These genes that make up this environmental resistome have the potential to be transferred to pathogens and indeed there is some evidence that at least some clinically relevant resistance genes have originated in environmental microbes (Wright, 2010).

ANCIENT ORIGIN OF ANTIBIOTIC RESISTANCE GENES

It is well-established documented that the antibiotic resistance is a long-evolved trait in prokaryotes and the diverse pool of resistant genes co-evolved with antibiotics in non-clinical (natural) environment much before these have been used in human therapy. Most of the antibiotic producer organisms carry the respective antibiotic resistance genes and both the resistance and the antibiotic biosynthesis pathway genes are usually found in the same gene cluster (Allen et al., 2010).

In the evolutionary scale many antibiotics or their structurally related precursors are believed to be as old as amino acids. It has been suggested that antibiotics are more than 500 million years old, dating back to the Cambrian period and probably evolved at the same time vertebrate fish emerged (Baltz, 2008). The components of the antibiotics are believed to be even older and the postulation is substantiated by the occurrence of non-protein amino acids of peptide antibiotics in meteorites and other primordial sources (Johnson et al., 2008). Julian Davies at the University of British Columbia proposes that antibiotics could be some of the oldest biomolecules (Amábile-Cuevas, 2003).

Metagenomic analyses of ancient DNA from 30,000-year-old to Beringian permafrost sediments dating back to the late Pleistocene age demonstrated the presence of different antibiotic resistance genes encoding resistance to β-lactam, tetracycline, and glycopeptide antibiotics (D'Costa et al., 2011). Structural and functional studies of one of these ancient resistance genes, the *vanA*

(vancomycin resistance operon) that encodes an ATP dependent D-alanyl-D-lactate ligase confirmed that it is essentially indistinguishable from VanA ligase associated with the recently discovered vancomycin resistance in the clinic.

Very recently, Lechuguilla Cave in New Mexico, cut off from the human activity for over four million years, was found to harbor culturable microbiomes, highly resistant to antibiotics. Some strains were found to be resistant to as many as 14 different commercially available antibiotics. Resistance was detected to a wide range of structurally different antibiotics including daptomycin, an antibiotic of last resort in the treatment of drug resistant Gram-positive pathogens. Enzyme-mediated mechanisms (e.g., glycosylation, phosphorylation) leading to resistance against both natural and semi-synthetic antibiotics were also discovered in some ancient bacteria. Characterization of macrolide kinase obtained from one of these resistant organisms revealed it to be related to a known family of kinases circulating in drug resistant pathogens at present (Bhullar et al., 2012).

In another study, molecular analysis of a metagenomic library from the cold-seep sediments of the deep sea Edison seamount (about 10,000 years old) also demonstrated the presence of TEM-type ESBLs (TEM-1 and TEM-116) suggesting that β-lactam resistance in microorganisms is likely to be present prior to the modern antibiotic era, and the diversity of TEM β-lactamases is not a recent phenomenon, rather it is the result of a very ancient evolution (Song et al., 2005). Therefore, despite the fact that increase in the use of an antibiotic is directly associated with increase in the frequency of resistant strains, it is obvious that exposure to antibiotics is not a pre-requisite for the emergence of resistance. Even the diversification of the antibiotic resistance appears to occur much before the human intervention as evidenced by the occurrence of the TEM-type ESBL in the 10,000-year-old sediments of the Edison seamount. But the prevalence of the resistance bacteria having TEM-1 and TEM-116 was as low as 0.3% (25 of 8,823) and 0.06% (5 of 8,823), respectively (Allen et al., 2009). Recent investigations revealed that the origin of both antibiotic resistance and the biosynthetic genes date back to the ancient period much before the human civilization began. Both antibiotics and the resistance phenotype existed together in nature for a long time.

There are many other cases of occurrence of antibiotic resistance genes in apparently antibiotic-free environments. The metagenomic studies of remote Alaskan soil revealed the presence of divergent β-lactamase genes and a bifunctional β-lactamase (Allen et al., 2009). These β-lactamases are more closely related to ancestral homologs compared to those isolated in clinical settings and are capable of conferring resistance on *Escherichia coli* despite the evolutionary distance. In another example of antibiotic resistance in environments not impacted by antibiotics, the phenotype of more than 60% of the Enterobacteriaceae isolates from a pristine freshwater environment was found to be multidrug-resistant. An example of this evidence exists in the two strains of *Citrobacter freundii* that were collected prior to the antibiotic era (the 1920s) which carried ampC β-lactamase genes. The encoded AmpC β-lactamases were as active as the recent plasmid borne AmpC β-lactamases (class C) that were found in the antibiotic era (Barlow and Hall, 2002). Phylogenetic analysis of the genetically diversified serine β-lactamases suggests an ancient root for the antibiotic resistance genes (Hall and Barlow, 2003).

Even the Murray collection (procured before and after the introduction of antibiotics, i.e., between 1917 and 1952) exhibited ampicillin and tetracycline resistance in 11 out of 433 enterobacterial strains and the resistance was non-conjugative. These studies reveal that the determinants of antibiotic resistance existed in nature much before the human intervention. Millions of years ago, antibiotics and antibiotic biosynthetic pathways evolved suggesting that both antibiotics and the resistance genes are very ancient (Wright and Poinar, 2012). The presence of multidrug-resistant organisms even in this pristine environment reinforces the idea that the antibiotic resistome is ancient and omnipresent in the microbial genome.

CAN THE DOOMSDAY BE POSTPONED?

In keeping with a policy package recommended by WHO in 2002 emphasis was given on the availability of proper facilities to ensure rapid testing of antibiotic resistance, regular documentation and sharing of the surveillance data, uninterrupted access to quality medical service, regulation on the sale of antibiotics, development of new diagnostic tests, and novel antimicrobials. But the problem continues to be unabated till now (Leung et al., 2011).

Recently, scientists (Marraffini and Sontheimer, 2008) from the Northwestern University have discovered a specific DNA sequence, called a CRISPR (clustered, regularly interspaced, short palindromic repeat) locus, which confer prokaryotes a type of acquired immunity against the acquisition of resistance genes by encoding a sequence-specific defense mechanism against bacteriophages and thus conferring an immunity toward HGT. Members of recently potentially virulent strains of enterococcal have been shown to lack complete CRISPR loci. Expression of the CRISPR interference has also been found to prevent the transfer of antibiotic resistance and virulent genes, not only *in vitro*, but also *in vivo*, during a pneumococcal infection (Bikard et al., 2012). "If this mechanism could be manipulated in a clinical setting, it would provide a means to limit the spread of antibiotic resistance genes and virulence factors in *Staphylococcus* and other bacterial pathogens," hopes Erik Sontheimer, Associate Professor at the Weinberg College of Arts and Sciences.

The new Eco-Evo drugs and strategies target prevention of the evolution and emergence of resistant bacteria in the environment. This might be achieved by using inhibitors against four P's viz, penetration (by using 7-valent conjugated vaccine, PC7 to prevent colonization of resistant serotype in human), promiscuity (using broad host range conjugation inhibitor), plasticity (recombinase inhibitor), and persistence (decontamination of high risk clones or mobile genetic elements) of resistant bacteria. This strategy appears implementable in view of the fact that preventive measures recommended in various steps are mostly adoptable. However, their long-term impact on the environment warrants rigorous testing before implementation (Baquero et al., 2011). In order to combat the looming crisis, a number of different other approaches have been adopted. Pharmaceutical companies are tapping new sources (samples obtained from tropical rain forests, myxobacteria, marine bacteria, extremophilic bacteria) for novel antibiotics. Development of antimicrobials against bacterial molecules,

which were not targeted earlier (e.g., bacteria DNA polymerase III, the cell division protein FtsZ, fibronectin binding proteins) is aimed at. Genes indispensable for survival of pathogens are shortlisted in search of new targets. In another strategy, molecular techniques are being used to clone the antibiotic biosynthetic genes cluster of a strain into a different strain, ultimately to get a hybrid molecule with antibiotic activity. In another approach called directed evolution, libraries are developed with randomly mixed and matched gene clusters obtained from different antibiotic producer strains. These libraries are transformed into protoplasts and recombinants are screened for improved spectrum of antibiotic activity. Large number of new compounds synthesized on a solid support, are screened for activity in another technique called combinatorial chemistry. These new strategies are expected to generate new compounds with novel antimicrobial activities (Strohl, 1997).

Several secondary approaches like reduction of antibiotic consumption, preservation of existing therapeutics, development of new antibiotics, and development of new strategies like sensitization of pathogens (Toney et al., 1998) have been adopted. The formulation, augmentin, a combination of β-lactamase inhibitor (clavulanic acid) and amoxicillin, is being successfully used against a variety of penicillin-resistant pathogens.

CONCLUSION

It is apparent that antibiotics are endowed with multifaceted activities and the cell modulates their role. The dual nature of antibiotic acting both as signaling molecule as well as growth inhibitory molecule suggests that antibiotic resistance genes have to co-evolve with antibiotics to safe guard the producer organism from antibiotic threat.

While it is obvious that dissemination of resistance-conferring genes has been facilitated following introduction of antibiotics into the clinical practice, we are yet to get sufficient evidence to conclude that human intervention is the only contributing factor behind the spread of resistance. Until and unless we generate required amount of information to fill up the gap of knowledge in this aspect, we cannot expect to devise measures which would effectively arrest the progress of the crisis.

Antibiotic resistance is a complex and continually evolving problem. A vast body of information has been engendered during the past 70 years on the antibiotic resistance and its dissemination. Comparative genomics, molecular genetics, combinatorial chemistry, and structural biology are being applied to explore new antibiotics but still the struggle is on. Several secondary approaches like reduction of antibiotic consumption, preservation of existing therapeutics, development of new antibiotics, and development of new strategies like sensitization of antibiotic-resistant organisms using QS inhibitors have been adopted but without much success.

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. This process was very rapid on an evolutionary scale and HGT, mediated by mobile genetic elements, played a prominent role in it. So it would be wise to plan a strategy to control the process of HGT to control subsequent transfer of resistance genes to pathogens.

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Broad-host-range IncP-1 plasmids and their resistance potential

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The plasmids of the incompatibility (Inc) group IncP-1, also called IncP, as extrachromosomal genetic elements can transfer and replicate virtually in all Gram-negative bacteria. They are composed of backbone genes that encode a variety of essential functions and accessory genes that have implications for human health and environmental bioremediation. Broad-host-range IncP plasmids are known to spread genes between distinct phylogenetic groups of bacteria. These genes often code for resistances to a broad spectrum of antibiotics, heavy metals, and quaternary ammonium compounds used as disinfectants. The backbone of these plasmids carries modules that enable them to effectively replicate, move to a new host via conjugative transfer and to be stably maintained in bacterial cells. The adaptive, resistance, and virulence genes are mainly located on mobile genetic elements integrated between the functional plasmid backbone modules. Environmental studies have demonstrated the wide distribution of IncP-like replicons in manure, soils and wastewater treatment plants. They also are present in strains of pathogenic or opportunistic bacteria, which can be a cause for concern, because they may encode multiresistance. Their broad distribution suggests that IncP plasmids play a crucial role in bacterial adaptation by utilizing horizontal gene transfer. This review summarizes the variety of genetic information and physiological functions carried by IncP plasmids, which can contribute to the spread of antibiotic and heavy metal resistance while also mediating the process of bioremediation of pollutants. Due to the location of the resistance genes on plasmids with a broad-host-range and the presence of transposons carrying these genes it seems that the spread of these genes would be possible and quite hazardous in infection control. Future studies are required to determine the level of risk of the spread of resistance genes located on these plasmids.

Keywords: IncP plasmid, antibiotic resistance, heavy metals, xenobiotic, catabolism, horizontal gene transfer, bioremediation

CHARACTERISTICS OF IncP-1 PLASMIDS

A characteristic feature of all plasmids, including IncP-1 plasmids, is that they belong to specific incompatibility (Inc) groups. Plasmids are classified into Inc groups based on their replication and partitioning systems: two plasmids belonging to the same Inc group are unable to coexist in a single bacterial cell (Shintani et al., 2010b).

Members of the *Enterobacteriaceae* family and the genus *Pseudomonas* carry plasmids belonging to more than 30 Inc groups. Plasmids from four of these groups (IncP, W, N, and Q) can transfer between and maintain themselves in both enteric bacteria and strains of *Pseudomonas* (Dröge et al., 2000). In particular, IncP plasmids are widely distributed in Gram-negative bacteria, e.g., in *Escherichia coli*, *Pseudomonas* spp., *Klebsiella aerogenes*, and *Sphingomonas* (Thomas, 2000; Shintani et al., 2010a). It should be added that IncP-1 in the *Pseudomonas* plasmid classification corresponds to IncP in the *E. coli* plasmid classification (Thomas and Haines, 2004).

The plasmids of the subgroup IncP-1 are of considerable interest to both molecular biologists and environmentalists due to their highly efficient conjugative transfer and ability to replicate in a broad range of hosts (Shintani et al., 2010a). These plasmids demonstrate great diversity – a phylogenetic study has shown the presence of 45 backbone genes on IncP-1 plasmids, but only 33 are shared by all the plasmids that have been completely sequenced to date.

A detailed phylogenetic analysis and study of the amino acid sequence of protein TrfA, which initiates plasmid replication, have allowed the classification of group IncP-1 into six subgroups: -α, -β, -γ, -ε, -δ (Bahl, 2009), and -ζ (Norberg et al., 2011) and also into an unnamed subgroup (Pachulec and van der Does, 2010). Unpublished data also indicate the existence of a η subgroup (Sen et al., 2012). IncP-1 plasmids have been identified in clinical and environmental, phylogenetically very distant, host bacterial species from around the world. Bacteria carrying these plasmids have been isolated from soil in areas contaminated by industry, pig manure, wastewater of industrial origin, river sediment, and fresh water (Bahl, 2009; Norberg et al., 2011; Sen et al., 2012). Comparative analysis of the nucleotide sequences of plasmids belonging to the IncP-1 subgroups α and β demonstrated the high similarity of their gene organization and also led to the identification of adaptive genes called "load genes." The backbone

sequences of these plasmids are responsible for the regulation of replication, stable maintenance in a bacterial cell and efficient conjugative transfer. In plasmids of the IncP-1β subgroup, the Tra1 and Tra2 regions of the backbone, that mediate conjugative transfer, are always separated by regions of clustered restriction sites, which may represent hotspots for the integration of various determinants. These regions can be a site for the insertion of mobile genetic elements (MGE) carrying catabolic genes, and antibiotic and heavy metal resistance determinants. The capacity for MGE integration is highly variable among IncP-1 plasmids. Plasmids pJP4, pSS60, and pSS50, belonging to the subgroup IncP-1β, contain one site for the integration of catabolic genes in their plasmid backbone, which is located between the *oriV* and *trfA* system replication sequences. The number of insertions in the backbone varies among IncP-1 plasmids (Trefault et al., 2004). pB4 is the only plasmid known to have three insertion sites: catabolic genes grouped into an operon are located near the *oriV* sequence, and antibiotic resistance determinants are located at both ends of region Tra2 (Adamczyk and Jagura-Burdzy, 2003).

There are currently only three known representatives of the subgroup IncP-1δ, namely plasmids pEST4011, pIJB1, and pAKD4. Plasmid pAKD4 is regarded as the prototype of this subgroup because it is the only one to possess the complete backbone sequence. Like other IncP-1 plasmids, those of subgroup δ carry specific genes that are located on transposons integrated within the plasmid backbone (Sen et al., 2010, 2012).

More than 50 plasmids belonging to the subgroup IncP-1ε have recently been identified and characterized. These plasmids confer resistance to a broad spectrum of antibiotics. This is due to the presence of a class 1 integron that carries the gene *sul1* responsible for sulphonamide resistance at its 5′ end, plus gene cassettes carrying several determinants of resistance to other antibiotics (Heuer et al., 2012). Plasmids belonging to subgroup IncP-1ε exhibit great diversity (Sen et al., 2011).

ANTIBIOTIC RESISTANCE GENES PRESENT ON IncP PLASMIDS

The extensive use of antibiotics for the treatment of humans and animals, and to stimulate the growth of livestock, has led to the rapid emergence and spread of resistance determinants to a broad spectrum of antibiotic substances (Allen et al., 2010; Aminov, 2010; Popowska et al., 2010, 2012; Tello et al., 2012). These determinants are located mainly on MGE such as plasmids and conjugative transposons, which ensures their spread by horizontal gene transfer. Horizontal gene transfer involving the acquisition of foreign DNA by transduction, transformation, or conjugation, is quite common among bacteria and plasmids play a key role in this process (Aminov, 2011; Martinez, 2009; Stokes and Gillings, 2011). Among the plasmids conferring resistance to antibiotics, representatives of the incompatibility groups P, Q, N, and W have been identified. These are characterized by their wide host range, including pathogenic bacteria, and so pose a serious threat to human health (Dröge et al., 2000). Numerous studies have demonstrated that the prevalence of such plasmids in different environments is very high, e.g., in soil, surface waters, wastewaters, and natural fertilizers (Götz et al., 1996; Haines et al., 2006; Binh et al., 2008; Sen et al., 2011; Heuer et al., 2012).

There are four basic mechanisms of plasmid-encoded antibiotic resistance: (i) enzymatic hydrolysis of the antibiotic molecule, which results in inactivation of the compound, (ii) enzymatic modification of the antibiotic to prevent interaction with the cellular target, (iii) efflux, which removes the antibiotic from the interior of the cell via specific cell membrane pumps, and (iv) a reaction called "bypass," which is the formation of an alternative metabolic pathway that allows bypassing of the blocked stage and modification of the antibiotic target (Alekshun and Levy, 2007). The genes encoding the proteins responsible for these mechanisms are found on plasmids present in both clinical and environmental bacterial isolates (Aminov and Mackie, 2007; Allen et al., 2010; Wright, 2010).

Table 1 shows plasmids from IncP-1 group, well characterized in terms of phenotype and molecular mechanisms of observed resistance to antibiotics and heavy metals. Resistance plasmids classified within subgroup IncP-1α were first identified in hospital isolates of *Pseudomonas aeruginosa* and *K. aerogenes*. These were found to encode multi-drug resistance (MDR) against antibiotics including penicillin, kanamycin, and tetracycline (Adamczyk and Jagura-Burdzy, 2003). Using the exogenous plasmid isolation method Dröge et al. (2000) found resistance plasmids, including those belonging to the subgroup IncP-1α, in bacterial communities from activated sludge derived from wastewater treatment plant (WWTP). Twelve plasmids were identified carrying an antibiotic and heavy metal resistance determinant, and ten of these could be classified to group IncP. These studies confirmed the wide distribution of this Inc group in these environments.

To date, the complete nucleotide sequences of six resistance plasmids belonging to subgroup IncP-1α have been determined. These plasmids, RP4/RK2, pTB11, pBS228, pB5, pB11, and pSP21, show a high degree of similarity with respect to elements of their plasmid backbone, while they are distinguished by the nature of the genes located within their adaptive modules (e.g., ISTB11, ISSP21, Tn*402* derivatives, Tn*1*, Tn*7*). Plasmid pB5 contains an integron of class 1 carrying a resistance cassette with *aac*C1 and *aacA4* genes, which encode enzymes that provide resistance to kanamycin, gentamicin, and tobramycin (Szczepanowski et al., 2011). The IncP-1α plasmids contain different variants of the integron *aadA* gene cassette (*aadA1*, *aadA2*, *aadA4*, and *aadA5*), which encode streptomycin/spectinomycin-300-*O*-adenylyltransferases conferring resistance to the aminoglycosides streptomycin and spectinomycin (Tennstedt et al., 2003, 2005). The *aadA2* gene cassette is widely distributed, having been identified in the integrons of many bacteria belonging to at least 11 genera. Numerous studies have demonstrated that different environmental and pathogenic bacteria share a common pool of integron-specific resistance gene cassettes (Tennstedt et al., 2003, 2005).

In the case of plasmids pTB11 and pSP21, a class 1 integron carrying genes that determine bacterial resistance to aminoglycosides is located within transposon Tn*402*. In addition, these plasmids possess a region containing genes for tetracycline resistance (*tetA* and *tetR*; Tennstedt et al., 2005; Szczepanowski et al., 2011).

The IncP-1α plasmid pTB11 carries the *aphA-orf3* module, encoding aminoglycoside-30-phosphotransferase responsible for aminoglycoside resistance, that shares the highest degree of similarity with the corresponding gene of plasmid RP4, originating

Table 1 | General features of IncP-1 plasmids carrying resistance genes.

Plasmid	Size (kb)	IncP-1 subgroup	Resistances	Resistance genes	Detected transposons	Reference/accession No.
pAKD1	58,246	IncP-1β	Sp, Sm, Su, Hg	*aadA, sul1, merE*	Tn*21*-like transposon	Sen et al. (2011)/JN106164.1
pB2/pB3[#]	60,732/56,167	IncP-1β	Aminoglycosides, β-lactam, Cm, Su, Tc, quaternary ammonium compounds	*aadA2, bla*$_{NPS\_2}$*, cmlA1, sul1, tetA(C), tetR(C), qacEΔ1*	Tn-*tet*, Tn*402*	Heuer et al. (2004)/NC_006388.1
pB4	79,370	IncP-1β	β-lactam, tripartite multi-drug resistance (MDR) efflux system, Sm, Em, Chr	*bla*$_{NPS\_1}$*, strAB, mexCD-oprJ, chr*	Tn*5393c*, Tn*5719*	Dröge et al. (2000), Tauch et al. (2003) /NC_003430.1
pB5	64,696	IncP-1α	Sm, Tc, Km, Gm, Su, quaternary ammonium compounds	*aacA4, aacC1, tetA, tetR, aphA, sul1, qacEΔ1*	nd	Dröge et al. (2000); Szczepanowski et al. (2011)/NC_019020.1
pB6	58	IncP-1β	Tc, Sm, Sp, Cm, Su	nd	nd	Dröge et al. (2000)
pB8	57,198	IncP-1β	Sm, Sp, β-lactam, Su, quaternary ammonium compounds	*aadA4, oxa2, sul1, qacEΔ1, qacF*	Tn*5501*, "cryptic" Tn, Tn*402/* Tn*5090*, Tn*QAC*/(Tn*3* family), Tn*501*/Tn*21*	Schlüter et al. (2005)/NC_007502.1
pB10	64,508	IncP-1β	β-lactam, Su, Sm, Tc, quaternary ammonium compounds, Hg	*oxa2, sul1, strAB, tetA, qacEΔ1, mer*	Tn*5393c*, Tn*1721*, Tn*501*	Schlüter et al. (2003)/NC_004840.1
pB11	66,911	IncP-1α	Tc, Ap, Km, Hg	*tetA, tetR, aphA, merE*	Tn*501*, Tn*5053*	Dröge et al. (2000), Szczepanowski et al. (2011)/CP002152.1
pB12	64,393	IncP-1β	Tc, Sm, Sp, Em, β-lactam / Su, quaternary ammonium compounds	*tetA, aacA4, oxa2, sul1, qacEΔ1*	Tn*21*, Tn*402*	Dröge et al. (2000), Sen et al. (2012) /JX469826.1
pTB11	68,869	IncP-1α	Aminoglycosides, β-lactam, Tc	*aphA, aadA1, aacA4, oxa2, tetA, tetR*	Tn*402/* (Tn*5090*), Tn*1721*,	Tennstedt et al. (2005)/NC_006352.1
pMCBF1	62,689	IncP-1ζ	Multi-drug efflux (MDE) outer membrane prot. NodT family, Hg	*oprN, merE*	Tn*5053*	Norberg et al. (2011)/AY950444.1
RP4/RK2	60,099	IncP-1α	Tc, Km, Ap	*tetA, aph*	Tn*4371*, Tn*1*	Pansegrau et al. (1994)/L27758.1
pTH10	70	IncP-1α	Tc, Km, Ap	nd	nd	Harayama et al. (1980)
R751	53,423	IncP-1β	Tp	*dhfrIIc*	Tn*402/* Tn*5090*, Tn*501*	Thorsted et al. (1998)
pKJK5	54,383	IncP-1ε	Tc, Tp, aminoglycosides, Su, Sp, quaternary ammonium compounds	*tetA, tetR, dfrA1, aadA11b, sul1, qacEΔ1*	Tn*402*	Bahl et al. (2007)/NC_008272.1

(Continued)

Table 1 | Continued

Plasmid	Size (kb)	IncP-1 subgroup	Resistances	Resistance genes	Detected transposons	Reference/accession No.
pG527	80,762	IncP-1α	Aminoglycosides, Km, Sm, β-lactam, Tc	*aadA1, aphA, sph,* bla$_{TEM-67}$, *tetA, tetR*	Tn*3*, Tn*7*, Tn*1721*	Sen et al. (2012)/JX469830.1
pSP21	72,683	IncP-1α	Tc, Km, aminoglycosides, β-lactam	*tetA, tetR, aph, aadA1, aacA4, oxa2*	Tn*402*	Pansegrau et al. (1994), Szczepanowski et al. (2011)/NC_019021.1
pBS228	89,147	IncP-1α	Aminoglycosides, Sp, Tp, Tc, β-lactam, Hg	*aadA, dhfr, tetA,* bla$_{TEM-67}$, *aph, merE*	Tn*1013*, Tn*5718*, Tn*1*, Tn*7*	Haines et al. (2007)/NC_008357.1
BRA100	56,265	IncP-1β	Aminoglycosides, Su, quaternary ammonium compounds, Hg	*aacA4, strAB, sul1, qacEΔ1, merE*	Tn*6305*/(Tn*3* family)	Unpublished/CP003505
pWEC911	74,056	IncP-1	Tc, β-lactam, Km, Hg	*tetA,* bla$_{TEM-67}$, *aphA, merE*	nd	Sen et al. (2012)/JX469833.1
pKSP212	54,342	IncP-1	Aminoglycosides, Su, *Hg*	*aac(6)-Ib, sul1, merE*	Tn*3*	Sen et al. (2012)/JX469831.1
pBRSB222	36,880	IncP-1	Aminoglycosides, β-lactam, Su, quaternary ammonium compounds	*aadA5, oxa2, sul1, qacEΔ1*	nd	Sen et al. (2012)JX469825.1
pKS208	50,604	IncP-1	Aminoglycosides, Km, β-lactam	*aac(6)-Ib, aphA1, pEC-IMPQ_139*	Tn*1525*, Tn*5053*/Tn*402*	Sen et al. (2012)/JQ432564.1

Ap, ampicillin; Cb, carbenicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nx, nalidixic acid; Sm, streptomycin; Sp, spectinomycin; Su, sulfanilamide; Tc, tetracycline; Tp, trimethoprim; Tm, tobramycin; Hg, inorganic mercury; nd, not determined.

[#] *Plasmids pB2 and pB3 differ only by a duplication of a tetA(C)-tetR-tnpAIS26 fragment in pB2.*

from a clinical isolate. Another aminoglycoside resistance gene cassette located on pTB11 and pSp32 encodes an aminoglycoside-60-*N*-acetyltransferase mediating resistance to different aminoglycosides such as tobramycin, amikacin, and gentamicin. Two other aminoglycoside resistance gene cassettes, *aadB* and *aacA29b*, which respectively, code for an aminoglycoside-200-*O*-adenylyltransferase and an aminoglycoside-60-*N*-acetyltransferase, have also been identified on IncP-1α plasmids. The *aadB* gene was found on a plasmid from a clinical isolate of *Acinetobacter baumannii*, while an *aacA29b* gene cassette, present on a plasmid, represents a new variant of *aacA29a* previously found on In59 in a clinical *P. aeruginosa* isolate (Siarkou et al., 2009).

Resistance plasmids are also represented in subgroup IncP-1β. These include the fully sequenced plasmids pB2/pB3, PB4, PB8, and PB10. These plasmids carry a class 1 integron or, in the case of PB4, a fragment of Tn*402*. Besides *sul1* that confers resistance to sulfonamides, these plasmids may also contain genes for resistance to other antibiotics and chemotherapeutics, e.g., *oxa* (PB8, PB10, and pB11) and *bla* (PB4 and PB3), encoding β-lactamase enzymes responsible for resistance to β-lactam antibiotics. Parts of the *oxa1* gene cassette in plasmid pSp33 and *blaNPS1* on the Tn*402* remnant in this plasmid are nearly identical to corresponding regions identified in different Korean clinical isolates of *Salmonella enterica* ssp. *enterica* (Lee et al., 2003, 2004) and *P. aeruginosa* (Jacoby and Matthew, 1979; Pai and Jacoby, 2001), respectively. However, some *bla* genes identified in this subgroup of plasmids have novel sequences. All of the aforementioned IncP-1β plasmids also carry genes encoding resistance to streptomycin, a member of the aminoglycoside antibiotic family. Plasmids PB4 and PB10 contain transposon Tn*5393c*, which includes the genes *strA* and *strB*, encoding aminoglycoside-3′′-*O*-phosphotransferase and aminoglycoside-6-*O*-phosphotransferase, respectively, that modify the streptomycin molecule, thus causing its inactivation. Identical or almost identical copies of Tn*5393* were recently identified in different human, fish, and plant pathogens. Plasmid pB10 also possesses the tetracycline resistance transposon Tn*1721*, that was first described in *E. coli* (Allmeier et al., 1992). A very similar transposon has been found in conjugative plasmids from the fish pathogen *Aeromonas salmonicida* (Sørum et al., 2003), the human pathogen *Salmonella enterica* ssp. *enterica* (Chiu et al., 2005) and a clinical isolate of *E. coli* (Boyd et al., 2004). With the use of plasmid pB10, carrying amoxicillin, streptomycin, sulfonamide, and tetracycline resistance genes, is has been shown that this broad-host-range IncP-1 plasmid can be transferred to foodborne pathogens (*Salmonella* spp. and *E. coli* O157:H7) under laboratory conditions (Van Meervenne et al., 2012). These results show that the antibiotic resistance genes encoded on plasmid pB10 can be expressed in the new hosts (pathogenic organisms) making these pathogens resistant to multiple antibiotics. These studies have shown that there is a serious risk to human health in cases of such transfer. The plasmid PB3 carries the gene *clmA1* conferring resistance to chloramphenicol. The product of the *clmA1* gene is a protein that acts as an efflux pump, which removes the drug from the bacterial cell (Schlüter et al., 2007). The genetic modules on these IncP-1β plasmids consist mainly of MGE that carry diverse determinants conferring resistance to different aminoglycosides, β-lactams, macrolides, sulphonamides, tetracycline, trimethoprim, disinfectants, and mercury ions. The greatest MGE diversity has been observed in plasmids of this subgroup isolated from bacteria living in WWTP (Szczepanowski et al., 2004). WWTP bacteria represent a reservoir for β-lactamase genes that have yet to be isolated from clinical strains. Mobile integron gene cassettes seem to function in the dissemination of β-lactamase genes in sewage habitats.

An important role in the spread of resistance genes in agricultural systems is played by representatives of subgroup IncP-1ε. The study of Heuer et al. (2012) showed that fifty plasmids belonging to this subgroup, isolated from samples of soil and manure, contain integrons of class 1 with gene cassettes plus the gene *sul1* (sulphonamide resistance). The nucleotide sequences of four of these plasmids have been determined (pKS77, pHH3414, pHH128, and pHH3408) and they differ mainly in the organization of the elements in transposon Tn*402*. These elements are a class 1 integron IS1326 and, in the case of plasmids pHH3414, pKS77, and pHH128, transposon Tn*1721* carrying the tetracycline resistance genes *tetA* and *tetR*. The resistance integrons contain the gene cassettes *aadA*, (pHH3414), *aadB* (pKS77), *aadA1b*, *dfrA1b*, and two copies of *catB* (pHH128; Heuer et al., 2012). The different *aad* gene variants encode adenyltransferases that modify the streptomycin and spectinomycin molecules, resulting in their inactivation. A similar mechanism is employed by the chloramphenicol acetyltransferase encoded by the *cat* genes to confer resistance to chloramphenicol. The *dfr* gene permits bacterial cells to survive in an environment with high concentrations of trimethoprim (Schlüter et al., 2007).

Interestingly, many studies have demonstrated a close relationship between the occurrence of MDR and resistance/tolerance to heavy metal ions (Lazar et al., 2002). Bacterial strains isolated from agricultural soil receiving wastewater can exhibit high levels of both metal and antibiotic resistance. Shafiani and Malik (2003) isolated 64 bacteria belonging to the genera *Pseudomonas*, *Azotobacter*, and *Rhizobium* from wastewater-irrigated soil. All the *Pseudomonas* isolates were resistant to heavy metals and cloxacillin, and more than half were also resistant to methicillin. Among these strains were MDR isolates. Ansari et al. (2008) found that 40 strains isolated from agricultural soils irrigated with wastewater showed resistance to most of the tested heavy metals (Ni, Cu, Zn, Pb). Moreover, around 75% of these strains were resistant to tetracycline, 57.5% to doxycycline, and 50% to ampicillin and nalidixic acid. The plasmids isolated from these resistant strains all belonged to the IncP group. Out of 12 pAKD plasmids (IncP-1 group) isolated from Norwegian soils only one, pAKD1, encoded antibiotic resistance in addition to mercury resistance. This plasmid carries a MDR Tn*21*-like transposon with a class 1 integron (Sen et al., 2011). These data suggest heavy metals treatment might have co-selected for MDR genes. This is consistent with previous and present observations that pollution with heavy metals can cause a rise in the abundance of drug resistance genes (Baker-Austin et al., 2006; Seiler and Berendonk, 2012).

CONCLUSION

The increasing use of antibiotics in human and veterinary medicine and agricultural production systems has caused the

increasing development of high levels of and novel antibiotic resistances and the rapid global spread of antibiotic resistance genes (Aminov, 2009, 2010; Martinez, 2009; Schlüter et al., 2007; Sen et al., 2011). A consequence of the horizontal transfer of the genes located on MGE, especially on Broad-host-range plasmids is the rising detection and levels of resistant bacteria, particularly of MDR bacteria, both in clinical and natural environments (Dröge et al., 2000; Aminov and Mackie, 2007).

IncP-1 plasmids are characterized by their wide distribution in the environment and ability to efficiently replicate in the cells of diverse hosts. In addition, the presence of transposons carrying both antibiotic and heavy metal resistance genes on these plasmids permits the host bacteria to survive and multiply in environments contaminated with these compounds. The broad-host-range IncP-1 plasmids carry determinants for resistance to at least one heavy metal (Ni, Cd, Co, Cu, Hg, Pb, Zn), and antibiotics of different groups, i.e., tetracyclines, quinolones, aminoglycosides, sulfonamides, β-lactams, and chemotherapeutics. This fact is not surprising in light of recent data showing that heavy metals present in the environment might have co-selected for MDR genes (Seiler and Berendonk, 2012). The two main mechanisms of antibiotic resistance encoded on IncP-1 plasmids are mediated by efflux pumps and enzymatic modification or hydrolysis of the antibiotic molecule. IncP-1 plasmid-containing bacteria are also key players in the horizontal transfer of antibiotic resistance in agricultural systems. It has been suggested that the spread of multiresistant bacteria in soil, water, and WWTPs is mainly due to their possession of IncP-1 plasmids (Schlüter et al., 2003, 2005; Aminov and Mackie, 2007; Bahl et al., 2007; Ansari et al., 2008; Norberg et al., 2011; Sen et al., 2011).

Based on their DNA sequences, it can be concluded that the vast majority of these resistance genes are located on MGE such as transposons and/or integrons (Tn*402*-like transposon with a class 1 integron), embedded within these transposons. In addition, the presence of different combinations of MGE and integrons in IncP plasmids as well as of characteristic hot spots for the integration of accessory elements creates unlimited possibilities for rearrangements of the genetic material of these plasmids. It therefore appears that the spread of resistance genes would be possible and quite hazardous in infection control. Literature data show that many representatives of this group of plasmids carrying resistance genes have been identified in pathogenic bacteria of the genus *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, *Aeromonas*, and *Pseudomonas*. Of course, in order to determine the level of risk of the spread of resistance genes located on these plasmids extensive experimental studies are required. However, the demonstrated ability to transfer multiresistance pB10 plasmid, isolated from a WWTP, to the foodborne pathogens *Salmonella* spp. or *E. coli* O157: H7 with expression of most of the transferred resistance genes in these strains poses a real threat to the life and health of people (Van Meervenne et al., 2012).

Detailed analysis of IncP-1 plasmid genomes can shed light on the adaptive mechanisms underlying bacterial evolution and this information may be used to develop effective methods of bioremediation of contaminated land with xenobiotic and antibiotics and heavy metals. Intensive research on the biodegradation of compounds that are a serious threat to the environment has led to the isolation and characterization of bacterial strains that can use these substances as a source of carbon and energy. In many cases, the adaptation of these microorganisms is due to plasmids (mainly of Inc group P) that carry genes encoding enzymes involved in the degradation of xenobiotics. It is known, that the degradative plasmids (IncP-1, P-7, and P-9) contain catabolic genes, often arranged as operons, encoding enzymes required for the degradation and utilization of many toxic compounds including naphthalene, toluene, chlorobenzene, p-toluenesulfonate, 2,4-D, haloacetate, and atrazine (Williams, 2004; Shintani et al., 2010a,b). The majority of bacterial genes encoding enzymes of catabolic pathways are located on MGE such as plasmids, insertion sequences (e.g., as a form of composite transposons), transposons, integrons, genomic islands, and phage genomes (Top et al., 2002; Top and Springael, 2003; Nojiri et al., 2004; Springael and Top, 2004).

Theoretically, genetic modules present in this type of element (both in catabolic and resistance plasmids) may be used in the construction of genetically modified strains that are optimized for use in bioremediation and biotechnology (Vidali, 2001; Urgun-Demirtas et al., 2006; Singh et al., 2011). This is of great importance due to the fact that these antimicrobial agents are powerful inhibitors of xenobiotic biodegradation activities, mainly due to their bacteriostatic or bactericidal activities. However, before the broader implementation of such microorganisms, further research is required to thoroughly characterize them in order to limit the spread of antibiotic resistance genes.

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RND multidrug efflux pumps: what are they good for?

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Multidrug efflux pumps are chromosomally encoded genetic elements capable of mediating resistance to toxic compounds in several life forms. In bacteria, these elements are involved in intrinsic and acquired resistance to antibiotics. Unlike other well-known horizontally acquired antibiotic resistance determinants, genes encoding for multidrug efflux pumps belong to the core of bacterial genomes and thus have evolved over millions of years. The selective pressure stemming from the use of antibiotics to treat bacterial infections is relatively recent in evolutionary terms. Therefore, it is unlikely that these elements have evolved in response to antibiotics. In the last years, several studies have identified numerous functions for efflux pumps that go beyond antibiotic extrusion. In this review we present some examples of these functions that range from bacterial interactions with plant or animal hosts, to the detoxification of metabolic intermediates or the maintenance of cellular homeostasis.

Keywords: multidrug efflux pumps, host/bacteria interactions, plant/bacteria interactions, quorum sensing, antibiotic resistance, bacterial homeostasis, bacterial virulence

INTRODUCTION

Multidrug resistance (MDR) efflux pumps are relevant elements that contribute to both intrinsic and acquired resistance to toxic compounds in diverse life forms, including humans where they have a role in resistance to anti-cancer drugs (Wu et al., 2011), to bacteria, where they are involved in resistance to antibiotics (Webber and Piddock, 2003; Li and Nikaido, 2004, 2009; Poole, 2005, 2007). Unlike well-known horizontally acquired antibiotic resistance determinants, MDR efflux pumps are usually chromosomally encoded and the structural components of different systems are highly conserved in all members of a given bacterial species (Saier et al., 1998; Paulsen et al., 2001; Saier and Paulsen, 2001; Paulsen, 2003; Baquero, 2004; Andam et al., 2011). MDR systems are ancient elements, present in bacterial genomes long before the use of antibiotics for the treatment of human infections (Martinez et al., 2009a). This, along with their ubiquity in different organisms, suggests that the main function of these elements goes beyond providing resistance to antibiotics. The fact that quinolones, a family of synthetic antibiotics, constitute a common substrate of MDR efflux pumps supports this notion (Alonso et al., 1999; Hernandez et al., 2011b). These observations also suggest that the recent selective pressure imposed by the use of antibiotics is not the main evolutionary driver for MDR efflux pumps (Alonso et al., 2001; Martinez et al., 2009b).

Bacterial MDR efflux pumps can be grouped into five different structural families: the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily (Lubelski et al., 2007), the multidrug and toxic compound extrusion (MATE) family (Kuroda and Tsuchiya, 2009), the major facilitator superfamily (MFS) (Pao et al., 1998), the small multidrug resistance (SMR) family (Chung and Saier, 2001), and the resistance/nodulation/division (RND) superfamily (Murakami et al., 2006; Nikaido and Takatsuka, 2009; Nikaido, 2011). The activity of an efflux pump depends on the different types of energy source each system uses: ABC transporters are fueled by ATP hydrolysis; MFS, RND, and SMR use the proton-motive force and MATE transporters consist of Na^+/H^+ drug antiport systems (Piddock, 2006a).

The RND family includes several members that are relevant to antibiotic resistance in Gram-negative bacteria, whereas the MATE family has been mainly associated to resistance in Gram-positive microorganisms (Piddock, 2006a; Vila and Martinez, 2008). This review will focus exclusively on RND efflux systems.

The crystallographic analysis of AcrB, a model member of the RND family, revealed that this protein forms a homotrimer (Murakami et al., 2002; Nakashima et al., 2011), that associates into a tripartite complex along with an outer membrane protein (OMP, TolC) and a periplasmic membrane-fusion protein (MFP, AcrA; **Figure 1**). Usually the genes encoding for these proteins are found in a single operon, however, the gene encoding for the OMP can also be found elsewhere in the chromosome, as it happens with TolC in *Escherichia coli* (Koronakis et al., 2000, 2004); or is part of an operon encoding for a different efflux pump (**Figure 2**). The *Pseudomonas aeruginosa* RND efflux pump MexXY is an example of the latter, where the system uses the OprM porin encoded in the *mexAB-OprM* operon (**Figure 2**; Mine et al., 1999).

In this review we will address the different functional roles that RND efflux pumps may have in addition to mediating antibiotic resistance. Exhaustive information on structure, regulatory aspects, and antibiotic resistance can be found elsewhere (Saier et al., 1998; Paulsen et al., 2001; Saier and Paulsen, 2001; Paulsen, 2003; Webber and Piddock, 2003; Li and Nikaido, 2004, 2009; Poole, 2004, 2007; Piddock, 2006a; Blair and Piddock, 2009; Nikaido, 2009, 2011; Nikaido and Takatsuka, 2009).

Some of the most relevant roles so far identified include involvement in bacterial virulence (Piddock, 2006b), plant–bacteria

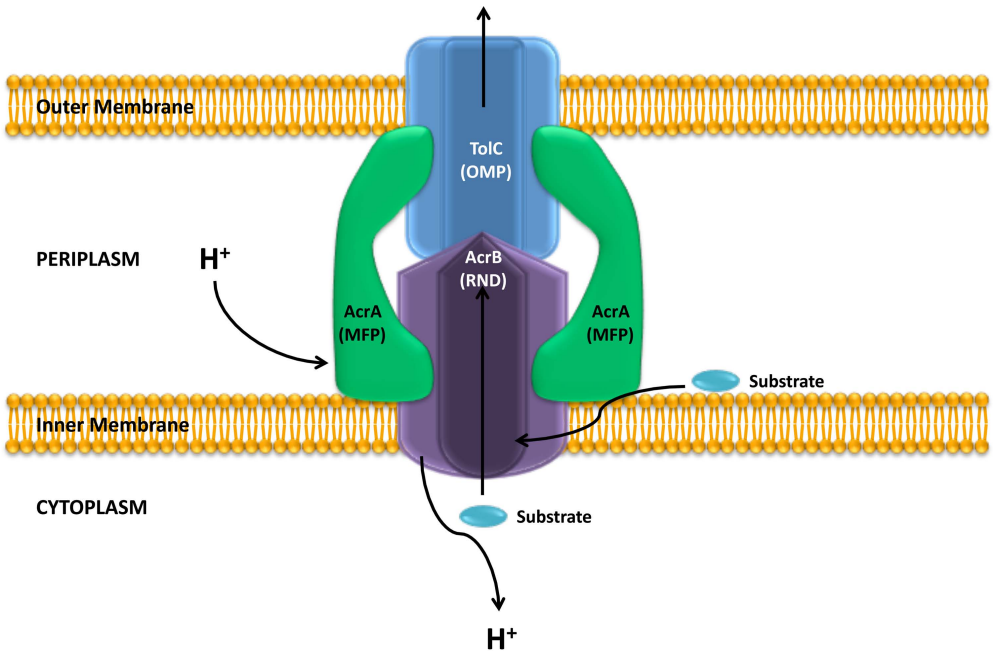

FIGURE 1 | Structure of an RND efflux pump. The figure shows a scheme of the structure of the *E. coli* AcrAB-TolC system. As shown, the system is a tripartite complex formed by the inner membrane AcrB protein, the outer membrane protein TolC and the membrane fusion protein AcrA. The activity of the AcrB RND protein is coupled to the proton gradient. It has been shown that these efflux pumps can extrude different compounds form the bacterial cytoplasm and the periplasm. Adapted from Blair and Piddock (2009).

interactions (Maggiorani Valecillos et al., 2006), trafficking of quorum sensing molecules (Evans et al., 1998; Kohler et al., 2001), and detoxification processes from metabolic intermediates, and toxic compounds such as heavy metals, solvents, or antimicrobials produced by other microorganisms (Aendekerk et al., 2002, 2005; Ramos et al., 2002; Nies, 2003; Sekiya et al., 2003; Burse et al., 2004a). A comprehensive review of all potential functions identified to date for all RND efflux pumps is beyond the scope of this review. Instead, we would like to discuss some selected examples of the ecological role that these systems may have in the absence of antibiotics. As stated above, we believe that the evolution of bacterial RND efflux pumps has been primarily driven by their physiological functions and not by the selective pressure imposed by the relatively recent human use of antibiotics. We consider the important role RND efflux pumps currently play in antibiotic resistance to be an evolutionary novelty stemming from the aforementioned use of antibiotics by humankind (Martinez, 2008; Baquero et al., 2009).

REGULATION OF RND EFFLUX SYSTEMS BY NATURAL EFFECTORS

The regulation of bacterial RND efflux systems is often mediated by global and local regulators, resulting in a multilayered control to optimize gene expression in response to specific cues. A number of positive and negative regulators along with their known mechanism of action have been reviewed elsewhere (Grkovic et al., 2002; Li and Nikaido, 2009).

In most cases a transcriptional regulator (typically a repressor) is encoded upstream the operon coding for the efflux pump (**Figure 2**). This local regulator usually keeps expression of the efflux pump at a very low-level. High-level expression can be achieved either through an effector-mediated release of the repressed state or through mutations in one or more regulators (Hernandez et al., 2009, 2011a). Activation may occur at different levels: (1) By inactivation of the local repressor that blocks the expression of the pump's structural genes such as AcrR in *E. coli* (Ma et al., 1996), MexR in *P. aeruginosa* (Poole et al., 1996; Sanchez et al., 2002c), or SmeT in *Stenotrophomonas maltophilia* (Sanchez et al., 2002a); (2) By activation of a global transcriptional regulator like SoxS, RobA, or RamA in *E. coli* (Martin et al., 2008; Zhang et al., 2008; Perez et al., 2012); (3) By switching on–off one or more steps that interlink regulatory cascades such as MtrR of *Neisseria gonorrhoeae* (Johnson et al., 2011); and (4) Through the emergence and selection of mutations in key genes like *mexT* in *P. aeruginosa* (Kohler et al., 1999).

Multidrug efflux pumps extrude a wide range of substrates. However, the number of effectors regulating them is lower in comparison. Understanding the mechanisms of regulation may help in deciphering the function of RND efflux pumps, since it is expected that different effectors trigger expression only when a given pump is required. RND efflux systems whose expression is controlled by natural inducers normally encountered during the course of infective processes have been studied in detail. Induction of expression by bile salts and fatty acids in enteric bacteria are perhaps the best studied examples of substances capable of modulating expression of these systems.

Expression of the *acrAB* system in *E. coli* is induced by decanoate and unconjugated bile salts usually encountered by the

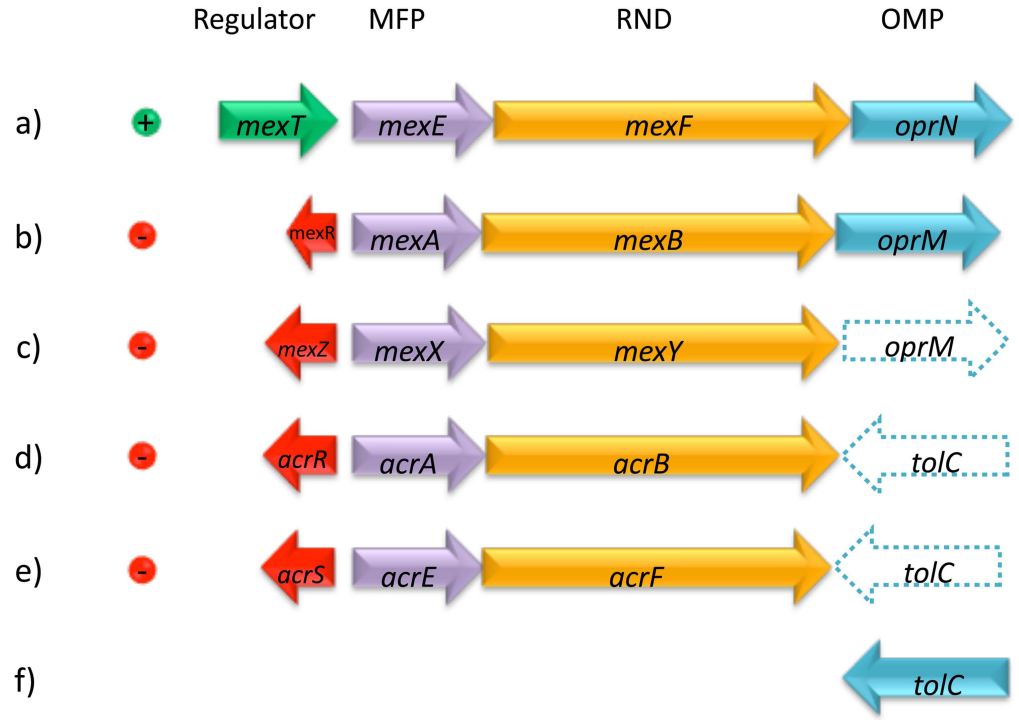

FIGURE 2 | Representative examples of transcriptional regulation and genetic organization of RND efflux systems. Local regulators can be either transcriptional activators, such as MexT (a) or transcriptional repressors, such as MexR, MexZ, AcrR, or AcrS (b, c, d, and e). The three structural components may be organized in a single operon, such as in the MexEF-OprN (a) or MexAB-OprM (b) systems; alternatively, a given system may use the OMP from another system, such as in the MexXY system using OprM (c). The OMP component may be located elsewhere in the chromosome, such as TolC (f) and can be used by one or more different systems as in the case of AcrAB (d) and AcrEF (e). MexEF-OprN, MexAB-OprM, and MexXY belong to *P. aeruginosa*; AcrAB and AcrEF belong to *E. coli*.

organism in the intestinal tract (Ma et al., 1995). The mechanism involves binding of these effectors to the Rob transcriptional regulator (Rosenberg et al., 2003). Bile salts also induce expression of *acrAB* in *Salmonella*, however, in this case the effector binds the RamA transcriptional regulator (Nikaido et al., 2008). Interestingly, in both cases the inductor is also a substrate for the efflux system, thus allowing the cell to respond quickly to deleterious environmental substances. Additional examples of bile salts-mediated induction include the *cmeABC* system in *Campylobacter jejuni*, the *vexD* gene in *Vibrio cholerae* and various RND-type efflux system genes in *Bacteroides fragilis* (Lin et al., 2005b; Pumbwe et al., 2007). These examples strongly suggest that these systems are relevant to bacterial adaptation for surviving in the gut and that this may be their original function.

In this regard, it has been suggested that some efflux pumps from human commensals and pathogens have evolved to overcome the innate immunity of the host (Blair and Piddock, 2009). For instance, the susceptibility to vertebrate antibacterial peptides in *N. gonorrhoeae* depends on the activity of the MtrCDE RND efflux pump (Shafer et al., 1998). Notably, this efflux pump is required to achieve mutation-driven resistance to penicillin in *N. gonorrhoeae* (Veal et al., 2002) and overexpression mutants present reduced susceptibility to several antibiotics and show an increase in *in vivo* fitness (Warner et al., 2007). MtrCDE (Jerse et al., 2003), enhances experimental gonococcal genital tract infections in female mice, whereas the FarAB-MtrE efflux pump (Lee and Shafer, 1999) is not needed to colonize this environment. It has been suggested that FarAB-MtrE is important for the resistance of *N. gonorrhoeae* to certain long-chained fatty acids that are present in the rectum (Lee and Shafer, 1999). Altogether these studies indicate that *N. gonorrhoeae* harbors efflux pumps each one responding to different environmental cues that enable adaptation for survival in different ecosystems.

Metal cations are another example of natural compounds capable of inducing expression of RND efflux pumps. Metals are required as cofactors in several bacterial processes. However, they are toxic at high concentrations. Consequently, bacteria harbor systems to maintain the cellular metal homeostasis. In some cases, this regulation implies that the efflux pump is involved in the extrusion of these toxic effectors (Nies, 2003). However, in other cases the situation is more complex and the effector is simply an environmental cue that indicates the type of ecosystem surrounding the organism. The *cusCBA* system in *E. coli* and *mtrCDE* system in *N. gonorrhoeae* constitute two of the most studied examples of metal-induced regulation among pathogenic bacteria. The CusCBA system confers tolerance to copper and silver ions (Franke et al., 2001; Grass and Rensing, 2001). Both substrates serve as natural inducers for *cusCBA* expression (Franke et al., 2001; Yamamoto and Ishihama, 2005), suggesting that this RND efflux pump may have been first selected to overcome the toxicity of these

metals. As stated above, the MtrCDE system is involved in resistance to host-derived antibacterial peptides (Shafer et al., 1998). It was recently reported that *mtrCDE* expression is indirectly regulated by free levels of iron through the regulation of its major transcriptional repressor, MtrR, by the MpeR transcriptional regulator (Mercante et al., 2012). Under the proposed model, expression of the efflux system would increase under iron-limited conditions, a situation that bacteria can encounter over the course of the infection process (Martinez et al., 1990). The *P. aeruginosa* CzcABC efflux system confers tolerance to zinc, cadmium, and cobalt and constitutes another example of metal-induced expression. The regulation occurs through the metal-inducible CzcRS two-component system that is activated in the presence of the system's substrates or indirectly in the presence of copper (Caille et al., 2007; Dieppois et al., 2012).

THE ROLE OF EFFLUX PUMPS IN PLANT–BACTERIA INTERACTIONS

The rhizosphere is a complex ecosystem characterized by a high microbial activity that results in a bacterial population density that can be two orders of magnitude higher than in bulk soil (Matilla et al., 2007). The structure of the rhizosphere's microbiota is governed by the release of nutrients through plant root exudates and by the ecological relationships of the microorganisms present in this ecosystem. A transcriptomic analysis of *Pseudomonas putida* grown in the rhizosphere of maize revealed that the expression of different efflux pumps is induced in this ecosystem (Matilla et al., 2007), thus suggesting a relevant function for the colonization of this environment. Plant exudates have been identified as good effectors of RND efflux pumps, and it has been shown that these secondary metabolites bind regulators of RND efflux pumps such as TtgR (Alguel et al., 2007), the local repressor of the TtgABC system in *P. putida* (Teran et al., 2003). Some compounds produced by plants have antibacterial effects and it has been described the RND efflux pumps are required from the first steps of bacterial plant colonization (Espinosa-Urgel et al., 2000) to survival in plant tissues (Barabote et al., 2003), possibly due to their involvement in protection against these compounds. This is the case of *Erwinia amylovora*, the cause of fire blight disease in rosaceous plants (Eastgate, 2000). The plantlet toxic metabolites naringenin and phloretin are good inducers of the efflux pump *acrAB* in this bacterial species, and *E. amylovora acrAB* mutants are much less virulent that their wild-type counterpart (Burse et al., 2004b). A similar situation occurs in *Agrobacterium tumefaciens*. Coumestrol, an antimicrobial root-exudated flavonoid, is both a substrate and an inducer of expression of the *ifeABR* efflux system (Palumbo et al., 1998). The fact that this system is needed for effective root colonization indicates that it plays an important role in *A. tumefaciens* resistance to plant-produced antimicrobials.

Comprehensive analyses on *Erwinia chrysanthemi* RND efflux pumps revealed that each system may differentially contribute to host specificity. Mutants defective in each of the pumps were differently affected in their virulence in diverse hosts and the susceptibility to plant-produced antimicrobials was specific for each pump (Maggiorani Valecillos et al., 2006). As discussed in the case of *N. gonorrhoeae,* this suggests that each of the several efflux pumps encoded in the genome of a given bacterial species may have a different function. This adaptation does not rely exclusively on the extrusion of toxic antimicrobial plant exudates. For instance, salicylic acid, an important signaling molecule produced by plants (Loake and Grant, 2007), induces the expression of the *E. chrysanthemi* efflux pumps *acrAB* and *emrAB* (Ravirala et al., 2007). This indicates that RND efflux pumps are relevant elements mediating bacteria/plant interactions at different levels that include the response to toxic compounds, host specificity and interspecies signal trafficking. This functional role is not confined to plant-infective bacteria. Mutants of the mutualistic symbiont *Rhizobium etli* lacking the RmrAB efflux pump form fewer nodules on its host *Phaseolus vulgaris* than the corresponding wild-type strain (Gonzalez-Pasayo and Martinez-Romero, 2000). Similarly, the SmeAB efflux pump plays an important role in the nodulation competitiveness in *Sinorhizobium meliloti* (Eda et al., 2011). The effect of efflux pumps on plant–bacteria interactions can be host-specific. For instance, BdeAB from *Bradyrhizobium japonicum* is needed for the symbiotic nitrogen-fixation activity on soybean, but not on other host plants such as mung bean and cowpea (Lindemann et al., 2010).

THE ROLE OF EFFLUX PUMPS IN BACTERIAL VIRULENCE

From a clinical point of view, antibiotic resistance could be considered as a colonization factor since only those organisms surviving within a treated patient will be able to cause an infection (Martinez and Baquero, 2002). However, in this section we would like to address the direct role that RND efflux pumps play in the virulence of different human pathogens. As mentioned in a previous section, the expression of different RND efflux pumps is triggered by human-produced compounds, and they contribute to the colonization of different environments in the human host. Although the role of efflux pumps on virulence has been studied for several organism (Piddock, 2006b), only in a few cases comprehensive studies including different systems from a single bacterial species have been performed. Below we discuss some of these examples

Vibrio cholerae RND EFFLUX PUMPS AND VIRULENCE

Vibrio cholerae possesses six different operons encoding for RND-type efflux systems: *vexAB*, *vexCD* (*breAB*), *vexEF*, *vexGH*, *vexIJK*, and *vexLM* (Kitaoka et al., 2011). While different RND efflux systems often share an OMP, it is rather common that operons encode for a cognate OMP for each system (**Figure 2**). In the case of *V. cholerae,* it seems that all six different RND efflux systems operate with the same OMP, encoded by the *tolC* gene (Bina et al., 2008; Cerda-Maira et al., 2008).

During the course of *V. cholerae* infections, bacteria colonize primarily the small intestine, where they penetrate the mucus lining coating the intestinal epithelium. In addition to factors produced by the innate immune system, the intestinal environment is rich in substances such as bile salts and organic acids that are capable of inhibiting bacterial growth (Reidl and Klose, 2002). Predictably, four *V. cholerae* RND efflux systems have been implicated in *in vitro* resistance to bile salts and detergents similar to detergent-like molecules the organism is likely to encounter during colonization of the intestinal epithelium.

Susceptibility studies with single and multiple mutant combinations revealed that VexB has broad substrate specificity and that it is the primary RND efflux system responsible for resistance to bile salts *in vitro* (Bina et al., 2008). VexD, VexK, and VexH have also been implied in resistance to bile salts, which denotes redundancy among the different RND efflux systems (Taylor et al., 2012). Moreover, the expression of *vexD* is induced in the presence of bile salts (Cerda-Maira et al., 2008). The VexK and VexH contribution to bile salts resistance is only evident in a Δ*vexBD* double mutant background, which suggests a supportive role for VexK and VexH. However, as Taylor et al. (2012) point out, this hierarchy might be limited to their *in vitro* experimental conditions. In fact, the increasing attenuation levels displayed by combination mutants in *in vivo* colonization experiments (Δ*vexBDK* < Δ*vexBDH* < Δ*vexBDHK* < ΔRND), suggest that VexH plays a more relevant role than VexK during the infection process.

RND efflux systems are also required for optimal expression of the genes encoding for two of the most important *V. cholerae* virulence factors: cholera toxin (CT) and the toxin-coregulated pilus (TCP). A ΔRND mutant exhibited decreased transcription of the *tcpA* and *toxT* genes, the latter encoding for a transcriptional activator responsible for transcription of the genes encoding for CT, and a concomitant decrease in CT and TCP production (Bina et al., 2008). While VexB is able to complement this phenotype, a *vexBDHK* still exhibits a decrease in CT and TCP, thus suggesting a role for VexM and VexF in virulence factor production (Bina et al., 2008; Taylor et al., 2012).

The mechanism through which the *V. cholerae* RND efflux systems modulate the production of virulence factors has not been elucidated. However, it has been proposed that deletion of systems with redundant functions could lead to the accumulation of a low molecular weight molecule that normally functions as a negative effector molecule involved in fine-tuning the expression of the affected virulence factors (Taylor et al., 2012). *V. cholerae* inhabits aquatic environments where it normally grows associated with zooplankton or egg masses (Reidl and Klose, 2002). It is possible that some of the RND efflux systems have dedicated functions specific to this portion of the organism's life cycle. This may be particularly true for VexM and VexF, for which no function in resistance to bile salts and antimicrobials has been identified to date.

Mycobacterium tuberculosis RND EFFLUX PUMPS AND VIRULENCE

The *M. tuberculosis* genome possesses 13 different genes encoding for RND proteins (Cole et al., 1998). Several domains in these proteins are unique to mycobacteria and are thus designated as MmpL (Mycobacterial membrane protein Large). Four *mmpL* genes appear to be in operons also containing an *mmpS* gene. The latter are predicted to encode for proteins equivalent to the MFPs in other bacterial RND systems (Domenech et al., 2005).

In spite of the documented *M. tuberculosis* resistance against first and second line antimicrobial therapy, none of the RND systems have been associated with antibiotic efflux to date, the only possible exception being MmpL7, which is capable of conferring isoniazid resistance when overexpressed in *Mycobacterium smegmatis* (Pasca et al., 2005; De Rossi et al., 2006; da Silva et al., 2011). Moreover, deletion mutants created in 11 *mmpL* genes failed to exhibit significantly altered drug susceptibility in *M. tuberculosis* (Domenech et al., 2005).

The primary role of most MmpL proteins appears to be the transport of lipids to be incorporated on the cell envelope. The complex mycobacterial cell wall is composed of peptidoglycan, arabinogalactan, and mycolic acids, the surface of which is covered by non-covalently associated lipids that include trehalose monomycolate (TMM), trehalose dimycolate (TDM), sulfolipids, phenolic glycolipids, and phthiocerol dimycocerosates (PDIMs; Tahlan et al., 2012). These lipids play important roles in protection against host-derived toxic molecules, bear an immunomodulatory activity and contribute to *M. tuberculosis* pathogenicity (Neyrolles and Guilhot, 2011). Lipid transport functions have been ascribed to MmpL3, MmpL7, and MmpL8, and in some cases deletion mutants have demonstrated the contribution of additional MmpL proteins to host survival and pathogenicity.

The inability to create an *mmpL3* deletion mutant combined with its absence in transposon mutant collections suggests that this gene is essential to *M. tuberculosis* (Domenech et al., 2005; Lamichhane et al., 2005). A recent study aimed at identifying the target of a novel *M. tuberculosis* antibiotic found data that suggests that MmpL3 transports TMM out of the cell and that its inhibition prevents the incorporation of *de novo*-synthesized mycolic acids into the cell envelope (Tahlan et al., 2012).

MmpL7 is required for PDIM transport to the cell surface and was the first MmpL protein implicated in lipid transport in *M. tuberculosis* (Cox et al., 1999). In addition, MmpL7 appears to function as a scaffold for the PpsE polyketide synthase required for the final step of phthiocerol synthesis, thus coupling transport and synthesis (Jain and Cox, 2005). At least two different studies have determined that *mmpL7* mutants display an attenuation phenotype in murine virulence models (Cox et al., 1999; Domenech et al., 2005). MmpL8 has been implicated in the transport of the SL-N, a precursor of the SL-1 sulfolipid, with a similar mechanism to that of MmpL7 where synthesis and transport appear to be coupled (Converse et al., 2003; Domenech et al., 2004). *mmpL8* mutants also display attenuated lethality in murine virulence models (Converse et al., 2003; Domenech et al., 2004, 2005).

Domenech et al. (2005) determined that an *mmpL4* mutant has both impaired growth kinetics and impaired lethality in a virulence murine model. The same study determined that while an *mmpL11* mutant shows a growth pattern similar to that of the wild-type during the active growth phase, the mutant is attenuated during the course of chronic infections in an *in vivo* model. No substrate has been identified for these transporters. A role in heme uptake has been recently proposed for MmpL11 and such a function would be in line with the attenuated virulence phenotype observed with an *mmpL11* mutant (Tullius et al., 2011). Furthermore, a role in extrusion of host-derived antimicrobials similar to that observed for *V. cholerae* RND efflux systems cannot be ruled out for those MmpL proteins that appear to be involved in the *M. tuberculosis* infection process.

Helicobacter pylori RND EFFLUX PUMPS AND VIRULENCE

The gastric colonizer *Helicobacter pylori* possesses three different operons encoding for RND efflux systems (Tomb et al., 1997). Over the years the systems have received different nomenclatures that may often lead to confusion when revising the literature: hp0605–hp0607 is also referred to as *hefABC*; hp0969–hp0971 was originally denominated as *hefFDE* and is currently known as *cznABC*; finally, the system encoded by hp1329–hp1327 was originally named *hefIHG* and currently hp1329 and hp1328 are known as *czcA* and *czcB*, respectively, while hp1327 is known as *crdB.*

Bina et al. (2000) initially assessed *in vitro* and *in vivo* expression profiles of each system as well as the individual contribution to intrinsic antibiotic susceptibility. The study revealed that hp0607 (*hefC)* and hp0969 (*hefF)* are expressed both in *in vivo* and *in vitro,* while hp1329 (*hefI*) is only expressed *in vivo.* Knockouts in each system failed to identify a contribution to intrinsic antibiotic susceptibility with 19 different compounds. However, overexpression of selected components has been associated with antibiotic resistance and different studies revisiting the contribution of each system to antibiotic susceptibility determined that hp0607 (*hefC*) and hp0605 (*hefA*) are involved in intrinsic antibiotic resistance to diverse antibiotics (Kutschke and de Jonge, 2005; Liu et al., 2008; Hirata et al., 2010; Tsugawa et al., 2011).

H. pylori is exposed to bile salts resulting from reflux into the human stomach; bile salts have an inhibitory effect on *H. pylori* growth, yet the ability to thrive in the presence of a bile gradient suggests that this organism has bile resistance mechanisms in place (Worku et al., 2004; Shao et al., 2008). HefC was recently found to play a role in resistance to bile salts and ceragenins (synthetic bile salt derivatives with antimicrobial activity; Trainor et al., 2011). A *hefC* mutant exhibited increased susceptibility to deoxycholate, cholate, glycodeoxycholate, taurodeoxycholate, taurocholate and to ceragenin 11(CSA 11); while no changes in susceptibility were observed with mutants in the other two efflux systems. Moreover, HefC appears to have substrate specificity for bile salts, since no change in susceptibility was observed with detergents. Although direct efflux of bile salts through HefC has not been experimentally demonstrated yet, it is likely that this system contributes to *H. pylori* successful colonization of bile-containing environments.

During the course of gastric colonization, *H. pylori* is exposed to additional environmental stresses, including low pH gradients (4.0–6.0) and acid shock. Acidic environments impact the bioavailability of metals like iron and nickel, which play an essential role in bacterial metabolism. In addition, environmental metal fluctuations are expected to arise from damaged epithelium and diffusion from ingested food (Stoof et al., 2008). Maintaining a cytoplasmic metal homeostasis is crucial to bacteria, as excessive concentrations can lead to severe cellular damage. The other two *H. pylori* RND efflux systems are involved in metal efflux.

The system encoded by hp1327–1329 (*crdB, czcB,* and *czcA*) constitutes a novel copper efflux pump. Expression of hp1329 is induced in the presence of copper and growth of hp1327 and hp1328 mutants is inhibited in the presence of this metal (Waidner et al., 2002). The same study found that expression of hp1326 (renamed as *crdA*), encoding for a secreted protein, is strongly induced in the presence of copper and growth of an hp1326 mutant was also impaired in the presence of copper. hp1326 is transcribed as a monocistronic unit, but is believed to constitute a copper resistance system along with hp1327–1329. A follow up study revealed that copper-mediated expression of hp1326 requires the CrdRS two-component system (Waidner et al., 2005); the study did not address expression of hp1327–1229. Mutants lacking the CrdRS system are unable to colonize the stomach of mice (Panthel et al., 2003). This suggests that hp1326 and hp1327–1329 might play an important role during the infective process of *H. pylori.*

The RND efflux system encoded by hp0969–0971 (renamed as *cznABC*) has been implicated in cadmium, zinc, and nickel resistance (Stahler et al., 2006). Stahler et al. (2006) showed growth inhibition of individual mutants in the presence of these metals. The *H. pylori* urease, a nickel-containing enzyme, is an essential colonization factor that enables survival in acidic conditions. Urease activity and expression is regulated in response to nickel availability (van Vliet et al., 2001), accordingly, *cznC* and *cznA* mutants exhibited enhanced urease activity (Stahler et al., 2006). The authors propose that the *cznABC* system plays an important role in fine-tuning urease activity, as nickel efflux reduces activity, while cadmium and zinc efflux prevents inhibition of this enzyme. High urea concentrations are toxic at neutral pH, therefore, untimely activation of this enzyme resulting from perturbations in metals homeostasis can be detrimental to the cell (Meyer-Rosberg et al., 1996; Rektorschek et al., 1998). The inability of *cznA*, *cznB*, and *cznC* mutants to achieve gastric colonization in a gerbil animal model and the failure of a *cznA* mutant to survive in acidic conditions might be linked to urease activity (Bijlsma et al., 2000; Stahler et al., 2006).

EFFLUX PUMPS AND GLOBAL BACTERIAL PHYSIOLOGY

One of the putative functions of RND efflux pumps is detoxification from detrimental intermediates derived from bacterial metabolism (Neyfakh, 1997). Studies on this subject have been mainly performed using mutants that overproduce RND efflux pumps. It is conceivable that overexpression of these elements might cause a metabolic burden on bacterial populations (Martinez et al., 2007, 2011; Andersson and Hughes, 2010). Indeed, different publications have shown that overproduction of RND efflux pumps may impact bacterial physiology (Sanchez et al., 2002b; Ruiz-Diez et al., 2003; Alonso et al., 2004; Linares et al., 2005; Lertpiriyapong et al., 2012; Olivares et al., 2012). Moreover, the uncontrolled production of these elements can affect the ability of pathogenic bacteria to infect experimental animal models, seriously impairing their virulence (Cosson et al., 2002; Hirakata et al., 2002; Warner et al., 2007; Lertpiriyapong et al., 2012; Perez et al., 2012).

The energy expenditure required to constantly maintain the activity of an efflux pump could lead to a fitness reduction upon overproduction of these elements. However, our group has recently shown that overproduction of the *P. aeruginosa* MexEF-OprN efflux system does not produce a fitness cost as measured in classical competition tests, although it alters several physiological aspects, including elements relevant for *P. aeruginosa* virulence

such as Type III and Type VI secretion (Tian et al., 2009a,b; Olivares et al., 2012). Notably, this effect is specific to each pump and might be associated to their functional role, as overexpression of either MexAB-OprM of MexXY does not produce the same effect (Linares et al., 2005).

As mentioned before, efflux pumps might be involved in the elimination of endogenous toxic compounds. The *P. aeruginosa* MexGHI-OpmD efflux system might be implicated in the extrusion of anthranilate, a toxic intermediate of the *Pseudomonas* quinolone signal (PQS) synthetic pathway (Aendekerk et al., 2002, 2005; Sekiya et al., 2003), whereas MexEF-OprN extrudes kynurenine, another intermediate in the same pathway (Olivares et al., 2012). A recent study has shown that kynurenine and its derivatives have relevant effects in different human diseases, including modulation of the activation of glutamate and nicotinic receptors, the modification of the immune response in situations of inflammation and infection, and the generation and removal of reactive oxygen species (Stone et al., 2012). Any potential impact that the constant extrusion of kynurenine by a MexEF-OprN overexpression mutant may have on the pathogenic behavior of *P. aeruginosa* remains to be established.

Pseudomonas quinolone signal is one of the quorum sensing (QS) signals produced by *P. aeruginosa* (Mcknight et al., 2000). Strains overexpressing MDR efflux pumps capable of extruding QS signals or their intermediates are likely to be impaired in the QS response. Indeed, overexpression of MexEF-OprN impairs the QS response of *P. aeruginosa* (Kohler et al., 2001; Olivares et al., 2012). Previous studies also showed that MexAB-OprM likely extrudes the 3O12-HSL QS signal (Evans et al., 1998; Pearson et al., 1999), and that overproduction of this efflux pump reduced the expression of selected QS-regulated genes. The *P. aeruginosa* QS regulon comprises approximately 5% of this organism's genome (Schuster et al., 2003); including several genes involved in virulence. Expression of some of these genes might be energetically costly. However, once the QS signals reach a specific threshold, expression of the regulon is maintained. It has been suggested that being signal-blind can be a good adaptive strategy to avoid this energetic burden (Haas, 2006). Whether the efflux pump-mediated extrusion of QS signals may be beneficial to *P. aeruginosa* under specific conditions remains to be determined.

Efflux pumps may also compensate for the effects that other bacterial elements may have on the organism. This might be the case of *C. jejuni,* a leading cause of food-borne enterocolitis worldwide (Ruiz-Palacios, 2007). As an intestinal pathogen this bacterium must overcome the antimicrobial effects of the bile salts secreted into the intestinal tract (Hofmann and Eckmann, 2006). The RND-type efflux pump CmeABC confers resistance to a broad range of antibacterial substances including bile salts, fatty acids, and detergents (Lin et al., 2005a). On the other hand, it has been demonstrated that the type VI secretion system (T6SS) plays a key role in the colonization of the intestinal tract (Lertpiriyapong et al., 2012). The activation of the T6SS may enable bile salts to enter inside the bacterium through the open secretion channel (Bidlack and Silverman, 2004); and this can compromise bacterial viability and infective capability. Bile salts trigger the expression of the CmeABC efflux pump; which extrudes the bile salts immediately outside the cell thus alleviating the entrance through the T6SS (Lin et al., 2005b). The functional interaction between the T6SS and CmeABC might be crucial for intestinal colonization by *C. jejuni*, thus playing a key role in the virulence of this bacterial pathogen (Lertpiriyapong et al., 2012).

Given the integration of RND efflux systems in bacterial metabolic networks, it is not surprising that their regulation is also incorporated in global regulatory networks. Global regulators such as MarA, RamA, and SoxS can activate the expression of efflux pumps such as AcrAB-TolC in *E. coli* and in additional *Enterobacteriaceae* (Davin-Regli et al., 2008). Similarly, the pleiotropic regulator MgrA (Luong et al., 2006) controls autolysis, virulence, biofilm formation, and efflux pump activity in *Staphylococcus aureus* (Ingavale et al., 2003, 2005; Truong-Bolduc et al., 2003, 2005; Trotonda et al., 2008). The control of efflux pumps by this global regulator is specific for each pump. Increased expression of *mgrA in vivo* in a subcutaneous abscess model upregulates expression of the *norB* and *tet38* efflux pumps, whereas expression of *norA* and *norC* is downregulated (Ding et al., 2008). The relevance of these pumps for the *in vivo* growth of *S. aureus* has been studied; *norB* and *tet38* defective mutants present a growth defect in a mice abscess model and the phenotype was not attributable to a staphylococcal stress response (Deng et al., 2012).

MexT, the transcriptional activator of MexEF-OprN in *P. aeruginosa* (**Figure 2**), constitutes another example of global regulation. MexT regulates the expression of several *P. aeruginosa* genes (Tian et al., 2009a). A portion of this regulation is mediated by the activity of the pump through the extrusion of a precursor of the PQS QS signal, and the concomitant impairment of the QS response (Olivares et al., 2012). However, the expression of other genes is directly regulated by MexT (Tian et al., 2009a). A recent study demonstrated that MexT functions as a redox-responsive regulator (Fargier et al., 2012), indicating that it might be involved in controlling cellular redox homeostasis. The fact that a local transcriptional regulator of an efflux pump behaves as a global regulator further supports the involvement of these elements in general processes of bacterial physiology and not simply as a response to the presence of antibiotics in the environment.

CONCLUDING REMARKS

The emergence of antibiotic resistance in bacterial human pathogens is a very recent process in the evolutionary timescale. It is often assumed that resistance genes have been mainly originated in antibiotic producers where they play a detoxification role (Benveniste and Davies, 1973; Webb and Davies, 1993; Davies, 1997). However, in the few cases where the origin of resistance genes has been tracked, the original hosts are not antibiotic producers. The QnrA gene from *Shewanella algae* constitutes a prime example, as it confers resistance to quinolones, which are synthetic antibiotics (Poirel et al., 2005). This indicates that, at least in some cases, antibiotic resistance would be an emergent function that has been recently selected due to the use of antibiotics for treating infections (Martinez, 2008, 2009a,b; Baquero et al., 2009; Fajardo et al., 2009). As we have seen in this review, MDR efflux pumps also fall within this category, since they

exhibit multiple functions relevant to bacterial physiology in addition to mediating antibiotic resistance. A complete understanding of these functions is important in order to define the networks that connect antibiotic resistance with other basic physiological processes (Linares et al., 2010; Martinez and Rojo, 2011), both during the course of infections and in natural, non-clinical ecosystems.

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Extracellular DNA-induced antimicrobial peptide resistance mechanisms in *Pseudomonas aeruginosa*

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Extracellular DNA (eDNA) is in the environment, bodily fluids, in the matrix of biofilms, and accumulates at infection sites. eDNA can function as a nutrient source, a universal biofilm matrix component, and an innate immune effector in eDNA traps. In biofilms, eDNA is required for attachment, aggregation, and stabilization of microcolonies. We have recently shown that eDNA can sequester divalent metal cations, which has interesting implications on antibiotic resistance. eDNA binds metal cations and thus activates the Mg^{2+}-responsive PhoPQ and PmrAB two-component systems. In *Pseudomonas aeruginosa* and many other Gram-negative bacteria, the PhoPQ/PmrAB systems control various genes required for virulence and resisting killing by antimicrobial peptides (APs), including the *pmr* genes (*PA3552–PA3559*) that are responsible for the addition of aminoarabinose to lipid A. The *PA4773–PA4775* genes are a second DNA-induced cluster and are required for the production of spermidine on the outer surface, which protects the outer membrane from AP treatment. Both modifications mask the negative surface charges and limit membrane damage by APs. DNA-enriched biofilms or planktonic cultures have increased antibiotic resistance phenotypes to APs and aminoglycosides. These dual antibiotic resistance and immune evasion strategies may be expressed in DNA-rich environments and contribute to long-term survival.

Keywords: antibiotic resistance, antimicrobial peptides, biofilm, PhoPQ, PmrAB, *Pseudomonas aeruginosa*, immune evasion, extracellular DNA

SOURCE AND FUNCTIONS OF EXTRACELLULAR DNA

Extracellular DNA (eDNA) is released from dead plant or microorganisms and accumulates in soil, aquatic, and sediment environments (Dell'Anno and Danovaro, 2005; Vlassov et al., 2007; Pietramellar et al., 2009). Bacteria actively release or secrete DNA, or it is released during bacterial lysis and outer membrane vesicle formation (Chiang and Tolker-Nielsen, 2010). eDNA is known to accumulate in many Gram-negative and Gram-positive bacterial biofilms (Tetz et al., 2009; Chiang and Tolker-Nielsen, 2010).

Extracellular DNA is present in healthy body sites and fluids, such as the gastrointestinal tract, blood, milk, secretions, and likely on mucosal surfaces (Vlassov et al., 2007). During infection, eDNA can accumulate due to the heavy recruitment of host immune cells and the production of neutrophil extracellular traps (NETs), as discussed later. Chronic lung infections in persons challenged with cystic fibrosis (CF) are caused by polymicrobial biofilms that are adapted for long-term survival. The sputum from CF patients has very high concentrations of eDNA and is the reason for the use of human recombinant deoxyribonuclease (DNase) as a mucolytic treatment (Shak et al., 1990; Ranasinha et al., 1993). Inhaled DNase (Pulmozyme) has been shown to reduce sputum viscosity, inflammation, and exacerbations, as well as improve lung function and survival (Jones and Wallis, 2010; Konstan and Ratjen, 2012).

DNA IS A NUTRIENT SOURCE

Given the abundance of eDNA in the environment, it is not surprising that DNA has a significant influence on bacterial physiology and serves many functions for bacteria. eDNA has been shown to serve as a sole nutrient source of phosphate, nitrogen, and carbon for *Pseudomonas aeruginosa*, *Escherichia coli*, and *Shewanella* spp. (Finkel and Kolter, 2001; Palchevskiy and Finkel, 2006; Pinchuk et al., 2008). We identified a secreted DNase (EddB) that is produced in the presence of low DNA concentrations and under limiting phosphate conditions (Mulcahy et al., 2010). The EddB DNase is required for degradation of eDNA and utilization of DNA fragments or nucleotides as a sole source of carbon, nitrogen, and phosphate (Mulcahy et al., 2010). There is an alkaline phosphatase expressed upstream of the DNase, EddA, which may also be required for phosphorus acquisition from DNA. In *Shewanella oneidensis*, a secreted DNase (ExeM) with significant homology to EddB (34% identity) is also required for utilization of DNA as a nutrient source (Godeke et al., 2011). A number of intracellular ssDNA exonucleases have also been shown to be required for growth using DNA as a sole carbon course (Palchevskiy and Finkel, 2006). DNA uptake also facilitates lateral gene transfer (LGT) and integration of foreign DNA sequences into the genome. Palchevskiy and Finkel (2006) proposed that dsDNA was taken into the cell, similar to the process of DNA uptake for LGT, converted to ssDNA and then degraded by intracellular exonucleases upon entry into the cytoplasm.

DNA IS A BIOFILM MATRIX POLYMER

Extracellular DNA is required and primarily acts to facilitate attachment, aggregation, stabilization, and maturation of biofilm

formation (Chiang and Tolker-Nielsen, 2010). DNase treatment of young *P. aeruginosa* biofilms results in biofilm dissolution, but mature biofilms resist DNase treatment, indicating a role in early biofilm formation (Whitchurch et al., 2002). Accumulation of exopolysaccharide (EPS) in mature biofilms probably accounts for the inability to degrade mature biofilms with exogenous DNase. Mutant strains that accumulated less eDNA during biofilm formation were more destabilized by treatment with sodium dodecyl sulfate (SDS; Allesen-Holm et al., 2006), providing further evidence for a role in biofilm stabilization. Treatment of young biofilms with DNase impaired the development of the cap structures of mushroom-shaped biofilms (Barken et al., 2008). DNase treatment of biofilms formed by Gram-negative or Gram-positive bacteria reduces the biomass, which suggests that eDNA is a ubiquitous DNA polymer (Tetz et al., 2009). The exception to the rule is in *Caulobacter crescentus* where eDNA blocks biofilm formation by binding to the polar holdfast structure, which is required for irreversible attachment (Berne et al., 2010). eDNA has been shown to localize to specific regions of mushroom-shaped microcolonies formed by *P. aeruginosa* in flow-chamber biofilms. In mature microcolonies, eDNA localizes primarily to the stalk structure, at the boundary of the stalk and cap (Allesen-Holm et al., 2006). In unstructured peg-adhered biofilms, eDNA can be visualized throughout thin biofilms with no particular organization (Mulcahy et al., 2008). eDNA has also been shown to be present as a matrix component in biofilms formed *in vivo* during infection with *P. aeruginosa* (Mulcahy et al., 2011; van Gennip et al., 2012), *Haemophilus influenzae* (Jurcisek and Bakaletz, 2007), and *Bordetella* (Conover et al., 2011).

EXTRACELLULAR DNA TRAPS

Neutrophil extracellular traps were first described in neutrophils, but have since been identified in other immune cell types including eosinophils and mast cells (Brinkmann and Zychlinsky, 2012). NETs can kill Gram-positive and Gram-negative bacteria, fungi, parasites, and viruses (Brinkmann et al., 2004; Urban et al., 2006, 2009; Guimaraes-Costa et al., 2009; Saitoh et al., 2012). Although there are numerous antimicrobial neutrophil components embedded in NETs (Urban et al., 2009), bacterial killing is largely attributed to the antimicrobial activity of histones (Brinkmann et al., 2004). NET killing can be blocked by either dissolving the NET structure with DNase, or by the addition of neutralizing anti-histone antibodies, which block histone antimicrobial activity. The process of NETosis is a novel mechanism of trapping and killing bacteria, as well as limiting bacterial dissemination (Brinkmann and Zychlinsky, 2012; McDonald et al., 2012; Yipp et al., 2012), For the purpose of this review, it is important to note that NET formation during infection is likely a major contribution of DNA accumulation at the site of infection. NET formation has been observed in CF sputum and likely contributes to the accumulation of eDNA during chronic CF lung infections (Marcos et al., 2010; Manzenreiter et al., 2012). Neutrophils are among the first immune cells recruited to the infection site and most of the DNA in the CF lung is derived from neutrophils (Lethem et al., 1990). In plant roots, an eDNA barrier is produced that protects the root from infection and is analogous to eDNA traps of human immune cells (Hawes et al., 2011).

CATION CHELATION AND ANTIMICROBIAL ACTIVITIES OF DNA

The focus of our initial work was to test the hypothesis that the matrix polymers influence bacterial gene expression. While biofilm polymers are known to have several protective immune evasion functions, we wondered if the matrix polymers also drive unique gene expression profiles that contribute to the phenotypes of cells in biofilms. Our initial observation upon addition of purified DNA exogenously to planktonic cultures was that bacterial growth was inhibited at DNA concentrations greater than 5 mg/ml (Mulcahy et al., 2008). Due to the highly anionic character of DNA, we hypothesized that DNA was a cation chelator and indeed demonstrated that DNA efficiently binds divalent metal cations that including Mg^{2+}, Ca^{2+}, Mn^{2+}, and Zn^{2+} (Mulcahy et al., 2008). In addition, DNA has a rapid antimicrobial killing activity that can be neutralized by pre-incubating DNA with excess cations before exposure to bacteria (Mulcahy et al., 2008). As bacterial surfaces are highly negatively charged and consequently have high levels of Mg^{2+} and Ca^{2+} bound to the surface (Nicas and Hancock, 1980), we suspected that DNA chelated cations from surfaces and disrupted membrane integrity. Using fluorescence microscopy to monitor membrane integrity, we demonstrated that DNA causes major perturbations to the outer and inner bacterial membranes, leading to rapid cell lysis and death. In addition, cells treated with antimicrobial concentrations of DNA released small outer membrane vesicles. This result indicated that DNA can strip sections of outer membrane from the envelope, disrupting outer and inner membrane integrity, resulting in cell lysis. The membrane destabilizing effects of DNA are similar to that of known cation chelator ethylenediaminetetraacetic acid (EDTA). DNA appears to have a broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria (Mulcahy et al., 2008).

ANTIMICROBIAL PEPTIDE KILLING AND RESISTANCE MECHANISMS

Cationic antimicrobial peptides (APs) are short, amphipathic peptides with broad-spectrum antimicrobial activity produced by the immune systems of most forms of life (Hancock and Sahl, 2006). The mechanism of killing is primarily through membrane binding and disruption, although they also disrupt cytoplasmic processes (Hancock and Sahl, 2006; Kraus and Peschel, 2006). Host defense peptides are another class of short peptides that may not have direct antimicrobial activities, but are protective due to their ability to modulate the innate immune response (Hancock and Sahl, 2006). APs are positively charged and therefore interact with the negatively charged lipopolysaccharide (LPS) in the Gram-negative outer membrane surface. The hydrophobic character permits membrane integration, disruption, and ultimately cell lysis and death. Gram-negative and Gram-positive bacteria alter their membrane charge to resist peptide killing by producing modified phospholipids, LPS, or teichoic acid structures, whose negative charges are masked (Kraus and Peschel, 2006; Anaya-Lopez et al., 2012). Surface modifications that contribute to AP resistance include alanine-modified teichoic acids, highly acylated lipid A, as well as phosphoethanolamine and aminoarabinose-modified lipid A species (Kraus and Peschel, 2006; Moskowitz and Ernst, 2010; Anaya-Lopez et al., 2012). Collectively, these modifications prevent or limit peptide binding or entry and disruption of bacterial membranes. CF isolates of *P. aeruginosa* are known to

produce highly acylated lipid A species and aminoarabinose modifications on the 1- and 4′-phosphates of lipid A (Moskowitz and Ernst, 2010).

DNA-INDUCED EXPRESSION OF THE *pmr* OPERON

The *pmr* genes are required for the covalent addition of aminoarabinose to the 1- and 4′-phosphates of lipid A (Moskowitz and Ernst, 2010), which protects the outer membrane from AP treatment (Johnson et al., 2012), and is required for peptide resistance (Moskowitz et al., 2004; Lewenza et al., 2005). The *pmr* genes are regulated by the PhoPQ and PmrAB systems in *P. aeruginosa*, and in many other Gram-negative organisms including *Salmonella enterica*, *Klebsiella pneumoniae*, and *Yersinia pestis* (Macfarlane et al., 1999; Groisman, 2001; McPhee et al., 2006; Cheng et al., 2010; O'Loughlin et al., 2010). The *P. aeruginosa* PhoQ sensor responds to Mg^{2+} levels and is activated under Mg^{2+} limiting conditions, leading to increased expression of the *pmr* operon. In Mg^{2+}-rich conditions, the presence of sub-lethal exposure to APs also induces expression of the *pmr* operon (McPhee et al., 2003), although this adaptive resistance is controlled by the CprRS and ParRS two-component systems (Fernandez et al., 2012).

Although DNA prevented growth at higher concentrations, we examined the influence of sub-lethal concentrations of DNA on *pmr* gene expression. In planktonic cultures grown in Mg^{2+} rich media supplemented with DNA, we showed that DNA caused a concentration-dependent induction of the *pmr* operon (*PA3552–PA3559*) in *P. aeruginosa* (Mulcahy et al., 2008). DNA induction of this operon can be explained by cation sequestration by DNA, and subsequent activation of the PhoPQ/PmrAB systems. Increased amounts of DNA resulted in more Mg^{2+} sequestered and therefore increasingly higher levels of *pmr* gene expression. **Figure 1** depicts the cation chelating effects of DNA on the structure of LPS in *P. aeruginosa*. Gene induction by DNA can be prevented by the addition of excess cations in combination with DNA, confirming that the cation chelating activity of DNA can be neutralized. We have recently shown that eDNA can also induce expression of the *Salmonella enterica* serovar Typhimurium *pmr* operon and causes increased AP resistance (Submitted), indicating that eDNA may play a general role in activating the PhoPQ system in DNA-rich environments.

DNA-INDUCED EXPRESSION OF SPERMIDINE SYNTHESIS GENES

A large number of *P. aeruginosa* genes are regulated under Mg^{2+} limiting conditions; some exclusively by PhoPQ and others are controlled by a second Mg^{2+} sensing two-component system PmrAB (McPhee et al., 2006). While the *pmr* operon is directly controlled by both PmrA and PhoP (McPhee et al., 2003, 2006), we identified a three-gene cluster upstream of PmrAB with homology to spermidine synthesis genes *PA4773* (*speD*) and *PA4774* (*speE*) that is controlled exclusively by PmrAB (McPhee et al., 2003). The addition of DNA to planktonic cultures also induced the expression of *PA4773–PA4775* in a concentration-dependent manner (Johnson et al., 2012). Mutants in the *PA4773–PA4775* genes were sensitive to APs, indicating a potential role in resistance to APs (Lewenza et al., 2005). We confirmed that *PA4773–PA4774* were required for spermidine synthesis, which is localized on the bacterial surface (Johnson et al., 2012). Surface and exogenous spermidine protects the outer membrane from APs polymyxin B and CP10A, but also from treatment with other cationic antibiotics including the aminoglycoside gentamicin (Johnson et al., 2012). Polyamines are typically found in the cytoplasm but here we have identified a novel role for polyamines on the bacterial surface. In the presence of eDNA, we proposed that *P. aeruginosa* produces spermidine as an organic polycation replacement for the divalent metal cation Mg^{2+} that functions to mask the negative surface charge and block AP binding (**Figure 1**). Magnesium ions are essential to cross-bridge the core phosphates of lipid A, so it is not surprising that *P. aeruginosa* produces a replacement polycation in the presence of DNA or under Mg^{2+} limiting conditions. Surface polyamines also act as antioxidants and quench reactive oxygen species, thereby protecting the outer membrane from oxidative stress damage to lipids (Johnson et al., 2012).

DNA-INDUCED ANTIBIOTIC RESISTANCE IN BIOFILMS

To test for a role of DNA-induced expression of the *pmr* genes in biofilm-specific antibiotic resistance, we determined the minimum biofilm eradication concentration (MBEC) in wild type biofilms and in biofilms formed the presence or absence of exogenous DNA (Mulcahy et al., 2008). DNA-enriched biofilms were shown to be eightfold more tolerant to the APs polymyxin B and colistin, and 64- to 128-fold more tolerant to the aminoglycosides gentamicin and tobramycin. Interestingly, planktonic cultures containing exogenous DNA also demonstrated DNA-induced resistance to aminoglycosides and APs (Mulcahy et al., 2008). Exogenous DNA did not have an effect on β-lactam or fluoroquinolones resistance. A mutant in the *pmr* cluster did not exhibit any DNA-induced resistance to APs, indicating that these genes were expressed and required for resistance in DNA-enriched biofilms (Mulcahy et al., 2008). The *pmr* mutant showed an intermediate aminoglycoside resistance phenotype, indicating that the *pmr* aminoarabinose modification also contributed partially to DNA-induced aminoglycoside resistance. It is possible that the anionic eDNA bound positively charged aminoglycosides and provided some protection as a matrix barrier, thus explaining the residual level of resistance in the presence of eDNA. It is known that DNA is capable of binding to aminoglycosides (Ramphal et al., 1988; Purdy Drew et al., 2009) and APs (Bucki et al., 2007). Therefore it is possible that DNA can induce specific resistance mechanisms and also act as a protective matrix absorbing and limiting antimicrobial exposure.

CONCENTRATION OF eDNA IN BIOFILMS AND INFECTION SITES

An important question that has not been fully answered is to determine if sufficient DNA accumulates in biofilms or during infections, to induce the expression of these protective, AP resistance phenotypes. In microarray studies comparing the gene expression profiles of biofilm to planktonic cultures, the PhoPQ/PmrAB-controlled genes are not among the biofilm-induced genes (Whiteley et al., 2001; Waite et al., 2005). This may be due to an insufficient accumulation of DNA in these particular biofilm model systems, and/or the presence of high Mg^{2+} levels in the growth media used, which can neutralize eDNA and prevent activation of the Mg^{2+} sensing PhoPQ and PmrAB systems. However, a recent paper described a novel regulator of biofilm

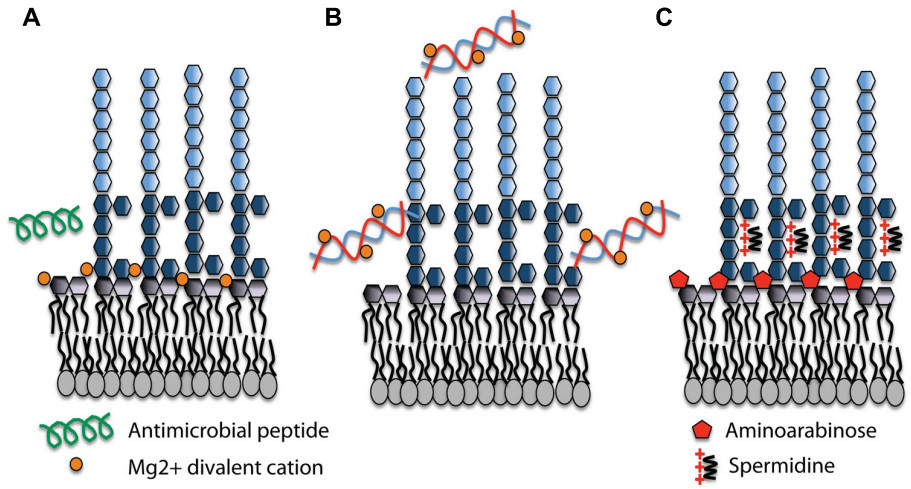

FIGURE 1 | Lipopolysaccharide (LPS) modifications in the presence of extracellular DNA that contribute to antimicrobial peptide resistance. **(A)** Divalent metal cations including Mg^{2+} (orange) bind to the negatively charged phosphates of the lipid A moiety of LPS and act to stabilize LPS. Antimicrobial peptides (green) can displace cations and disrupt membrane integrity, leading to cell lysis and death. **(B)** Extracellular DNA binds and sequesters cations from the environment and the membrane. **(C)** In response to limiting Mg^{2+} or cation chelation, the PhoPQ/PmrAB systems are activated leading to the production of covalently attached aminoarabinose to the phosphates of lipid A (red) and the production of polycation spermidine (charge, +3) on the surface, which may bind electrostatically to negative charges in the core oligosaccharide (dark blue) of the O antigen. Both modifications mask the negative charges and protect the outer membrane from peptide damage.

formation, BfmR, which is required for *P. aeruginosa* to transition to the maturation-1 biofilm developmental stage (Petrova et al., 2011). Biofilms formed by this mutant accumulated more eDNA, which was due to increased bacteriophage-mediated lysis in the *bfmR* mutant. Microarrays were performed on *bfmR* biofilms and both the *pmr* and *PA4774–PA4775* genes were induced in *bfmR* biofilms relative to wild type PAO1 (Petrova et al., 2011). This is likely due to the increased eDNA accumulation, but it may be possible that these genes are also controlled by BfmR.

Several papers have reported the *pmr-gfp* gene expression pattern in *P. aeruginosa* flow-chamber biofilms (Haagensen et al., 2007; Pamp et al., 2008). The *pmr* operon is required for colistin resistance in flow-chamber biofilms, but in many of these studies, there was little or no expression of the *pmr* operon in untreated biofilms. This result suggested that there is not sufficient eDNA accumulation in flow-chamber biofilms cultivated under these conditions to influence *pmr* expression. Shortly after colistin treatment, *pmr-gfp* expression was seen in a colistin resistant subpopulation formed on the caps of mushroom-shaped microcolonies (Haagensen et al., 2007). It is known that the presence of APs can induce the *pmr* genes, highlighting an adaptive resistance mechanism whereby the resistance genes are induced by exposure to sub-lethal concentrations of APs (McPhee et al., 2003). The colistin resistant subpopulation is metabolically active, motile, requires various multi-drug efflux pumps, and appears shortly after the early stages of surface attachment (Haagensen et al., 2007; Pamp et al., 2008). Colistin treatment was effective at killing the cells within the inner stalk structures but not the resistant subpopulation on the surface, indicating that colistin penetration is not limited in flow-chamber biofilms, despite the accumulation of eDNA and EPS in these biofilms (Haagensen et al., 2007; Pamp et al., 2008).

Although the total concentration of eDNA can be quantitated in biofilms (Wu and Xi, 2009), the localized concentration may be more important than the overall concentrations. The accumulation of DNA at infection sites is not well documented but sputum from the lungs of persons challenged with CF is known to accumulate DNA at concentrations ranging from <1 to 20 mg/ml (Shak et al., 1990; Ranasinha et al., 1993). There are relatively low Mg^{2+} concentrations in the CF lung (0.08–2 mM; Palmer et al., 2005; Sanders et al., 2006), not high enough to neutralize the cation chelating potential of such high DNA concentrations. Based on the known concentration of DNA and Mg^{2+} in CF lung, it is probable that the PhoPQ/PmrAB-controlled genes are expressed in the CF lung and may contribute to long-term survival in the CF lung. Recently, colistin resistant mutants have been characterized from CF patients and shown to contain gain-of-function PhoQ and PmrB sensor mutations, leading to increased expression of the *pmr* genes (Miller et al., 2011; Moskowitz et al., 2012). This result underscores the importance of these genes in the CF lung, particularly in those patients treated with colistin.

FUTURE WORK

To date, we have shown that eDNA influences the expression of several genes including a secreted DNase, and at least two operons controlled by the PhoPQ and PmrAB two-component systems. We are currently exploring the global effect of eDNA on bacterial gene expression using a genome-wide transcriptomic method and screening a library of transcription *lux* fusions (Lewenza et al., 2005) to identify novel DNA-induced or repressed genes. While aminoarabinose-modified LPS and surface spermidine both protect the outer membrane and contribute to AP resistance *in vitro*, they may also protect *P. aeruginosa* from APs produced by the innate immune system. It will be important to examine the role

of these surface modifications in protecting *P. aeruginosa* from innate immune cells known to produce APs, such as macrophages and neutrophils.

CONCLUSION

We identified a new property of eDNA as a divalent metal cation chelator, which is required to induce the expression of multiple operons that contribute to decreasing the permeability of the outer membrane to APs and aminoglycosides. *P. aeruginosa* EPS are also anionic polymers with calcium binding properties, indicating that cation binding and sequestration may be a general feature of the biofilm matrix. The anionic charge of DNA may also contribute to antibiotic resistance by binding to cationic antimicrobials and limiting their access to bacterial cells. Since DNA accumulates in the environment, in infection sites and in the biofilm matrix, the influence of DNA on gene expression may contribute to long-term survival in these DNA-rich environments.

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Concentration-dependent activity of antibiotics in natural environments

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Bacterial responses to antibiotics are concentration-dependent. At high concentrations, antibiotics exhibit antimicrobial activities on susceptible cells, while subinhibitory concentrations induce diverse biological responses in bacteria. At non-lethal concentrations, bacteria may sense antibiotics as extracellular chemicals to trigger different cellular responses, which may include an altered antibiotic resistance/tolerance profile. In natural settings, microbes are typically in polymicrobial communities and antibiotic-mediated interactions between species may play a significant role in bacterial community structure and function. However, these aspects have not yet fully been explored at the community level. Here we discuss the different types of interactions mediated by antibiotics and non-antibiotic metabolites as a function of their concentrations and speculate on how these may amplify the overall antibiotic resistance/tolerance and the spread of antibiotic resistance determinants in a context of polymicrobial community.

Keywords: antibiotic, resistance, tolerance, interaction, signal, stress, cue, community

INTRODUCTION

Antibiotics are bioactive small molecules naturally produced by secondary metabolism of microorganisms such as bacteria and fungi (Davies, 2006; Aminov, 2010; Davies and Davies, 2010). Their discovery as antimicrobial drugs has revolutionized the management and treatment of infectious diseases. Many easily treated infectious diseases today had high mortality rates in the pre-antibiotic era. However, due to the increasing prevalence of resistance, the *in vivo* efficacy of antibiotics is reduced or abolished, and the spread of antibiotic-resistant microorganisms is now threatening the treatment of otherwise manageable infections.

Antibiotic resistance in microbes is widespread and it has been demonstrated that soil bacteria are rich in resistance determinants to both natural and synthetic antibiotics commonly used in clinics (D'Costa et al., 2006). The collective genes that contribute to antibiotic resistance are referred to as the antibiotic resistome (D'Costa et al., 2007; Wright, 2007). Interestingly, antibiotic resistance genes extracted from the soil resistome were shown to be identical or highly similar to those found in clinically relevant drug-resistant human pathogens (Forsberg et al., 2012) demonstrating that lateral gene transfer likely plays a role in the rise of multidrug-resistant pathogens. In addition to the environment, the human microbiome is also a niche rich in antibiotic resistance determinants where exchange of resistance genes can lead to the generation of drug-resistant bacteria with potential pathogenic traits (Sommer et al., 2009, 2010; Sommer and Dantas, 2011). Altogether, these recent studies showed that the spread of antibiotic resistance from non-pathogenic environmental bacterial is an ongoing threat to the clinical use of antibiotics, even for new synthetic compounds, and the emergence of new drug-resistant pathogens is a constant threat.

Despite a better understanding of the different mechanisms leading to resistance, exposure to antibiotics is still considered the major driver in the selection for antibiotic-resistant bacteria (Levy, 2001; Marshall and Levy, 2011; Andersson and Hughes, 2012) and the selection occurs over a large spectrum of concentrations (Andersson and Hughes, 2012). Lethal concentrations of antibiotics rarely occur outside of therapeutic applications, but bacteria constantly face subinhibitory antibiotics in the environment and the host (e.g., human and other animals) following therapies. In fact, the release of antibiotics in the environment from medical or non-medical (e.g., agricultural) use artificially creates concentration gradients that are rarely encountered by environmental bacteria located in areas that are normally free of human-derived antibiotic activities (Aminov, 2009; Martinez, 2009a). The rapid appearance of drug-resistant bacteria upon antibiotic exposure implies that resistance and resistance mechanisms have co-evolved with antibiotic-derived products. The latter point raises the question as to whether antibiotic resistance was already a bacterial trait before the modern use of antibiotics. To address this question, elegant metagenomic and functional studies showed the existence of resistance determinants in pristine areas of the world (D'Costa et al., 2011; Bhullar et al., 2012) demonstrating that antibiotic resistance predates the clinical use of antibiotics. Therefore, antibiotic resistance is a common bacterial feature. The medical and non-medical use of antibiotics may accelerate the spread of resistance through positive selection in both the environment and the host.

The focus on the medical use of antibiotics has limited fundamental research regarding the other potential activities of these compounds in their natural settings, including the environment (e.g., soil) and hosts such as humans, animals, and plants. In complex communities containing antibiotic-producing microorganisms, bacteria are naturally exposed to lethal and non-lethal antibiotics making them trained at responding to these compounds. Non-lethal levels of antibiotics can alter the expression of

genes involved in a variety of bacterial functions like metabolism, regulation, virulence, DNA repair, and stress response (Goh et al., 2002; Tsui et al., 2004; Davies et al., 2006; Yim et al., 2006, 2007, 2011; Blazquez et al., 2012). Subinhibitory antibiotics can also modify cellular behaviors in bacteria with the formation of biofilms (Hoffman et al., 2005; Frank et al., 2007; Haddadin et al., 2010; Mirani and Jamil, 2011; Subrt et al., 2011; Kaplan et al., 2012) and persister cells (Dorr et al., 2010). Altogether, these observations strongly suggested that antibiotics induce responses other than those associated to their antimicrobial activities and it is now accepted that they might be used as "signaling" molecules with regulatory functions (Yim et al., 2007; Aminov, 2009; Allen et al., 2010).

Antibiotics are, like other bioactive small molecules, low molecular weight metabolites produced by secondary metabolism of microorganisms, i.e., are not considered essential for growth and viability. Microorganisms such as bacteria and fungi produce a wealth of small molecules that has been called the parvome (Davies, 2009; Davies and Ryan, 2012). Secondary metabolites are responsible for most of the interactions taking place in natural microbial communities (Lyon and Muir, 2003; Keller and Surette, 2006; Duan et al., 2009; Rath and Dorrestein, 2012) and as extracellular metabolites antibiotics have the potential to exhibit similar functions. Bacteria in natural environments are mostly part of complex polymicrobial communities in which all members share nutrient resources as well as chemicals including primary metabolic end products and secondary metabolites, which have the potential to induce antibiotic resistance/tolerance mechanisms. Natural communities also include host-associated communities such as the human microbiome. The role(s) of antibiotics in mediating non-lethal interactions between bacterial cells, may in fact, play a much bigger role than previously anticipated in the global antibiotic resistance threat that we are currently facing. Naturally occurring antibiotics, and other related secondary metabolites in bacterial communities, may dictate the spread of antibiotic resistance in a concentration-dependent manner. Therefore improving our current knowledge of antibiotic-mediated interactions in bacteria may facilitate the development of new therapeutic strategies for the treatment of drug-resistant pathogens. Here we review the current knowledge of antibiotic responsive activities as a function of their concentrations and speculate on how these antibiotic-mediated interactions may overall influence antibiotic resistance/tolerance and community composition in heterogeneous polymicrobial communities.

CHEMICAL INTERACTIONS

The explosion of research in cell–cell interactions mediated by bioactive small molecules in microbiology has led to the general assumption that most interspecies cell–cell interactions could be labeled as "communication" or "signaling." However, it is important to note that the demonstration of a biological response upon exposure to a chemical does not necessarily imply communication. The improper use of terms like signal, signaling, or communication in microbiology has created confusion since most interspecies metabolite-mediated interactions labeled as "signaling/communication" are often in conflict with evolutionary theories. A detailed analysis of the appropriate terminology is beyond the scope of this review; therefore we refer the reader to recent reviews that have thoroughly discussed the topic (Keller and Surette, 2006; Diggle et al., 2007; Stacy et al., 2012). A chemical mediating intra- or interspecies interactions can be defined as a signal, cue, or coercion (chemical manipulation). For a chemical interaction to occur, emitting bacteria must first produce a molecule that can be perceived by other individuals, and second, the receiver must alter its behavior in response to the signal.

To determine whether an interaction is mediated by a signal, a cue, or coercion the overall benefit of the reaction is used as primary criteria. As shown in **Table 1**, a signal is defined when both partners take advantage of the interaction (bidirectional), while cues or coercions have unidirectional benefits for receivers or emitters, respectively. In true signaling interactions, the production and detection of the signal have co-evolved specifically for that purpose and from an evolutionary perspective these events will only be maintained when both partners benefit from the information conveyed by the signal for which they evolved (Maynard Smith and Harper, 2003; Keller and Surette, 2006). On the other hand, a cue provides information to a receiver for which a response is triggered (Keller and Surette, 2006; Diggle et al., 2007; Stacy et al., 2012). Although not mediated by single molecules, environmental conditions can also be considered as cues by bacteria and they include pH, osmolarity, temperature, oxidative stress/oxygen, and nutrient limitation. The main distinction with a signal is that the biological response did not evolve for that purpose, which benefits only the receiver (Keller and Surette, 2006; Diggle et al., 2007; Stacy et al., 2012). Conversely, a coercion scenario is a strategy used by the emitter, via the release of a molecule, to chemically manipulate the receiver for its own benefit (Keller and Surette, 2006; Diggle et al., 2007; Stacy et al., 2012).

Figure 1 illustrates the different ways by which a single chemical can be perceived by bacterial cells of different species within a polymicrobial community. Although interactions mediated by signals, cues, or coercions can all occur in these communities, the majority of these dynamic interactions fall in the category of cue, because they do not require stability over time, for which only receiver cells evolve. The modulation of *Pseudomonas aeruginosa* virulence factors by autoinducer-2 (AI-2) from the oropharyngeal microbiota (Duan et al., 2003) and alteration of the antibiotic tolerance profile by volatile ammonia (Bernier et al., 2011) are examples of interspecies interactions mediated by cues.

Interestingly, intraspecies diversity may in some cases challenge the concept of true communication by the rise of cheaters through

Table 1 | Simplified description of chemical-mediated interactions[1,2].

	Benefits the emitter	Benefits the receiver
Signal	++	+
Cue	−	+
Coercion	+	−

[1] *The overall benefit is used as the main determinant for the classification of the different types of bacteria–bacteria interactions and are either beneficial (+) or costly (−).*
[2] *Adapted from Diggle et al. (2007) and Stacy et al. (2012).*

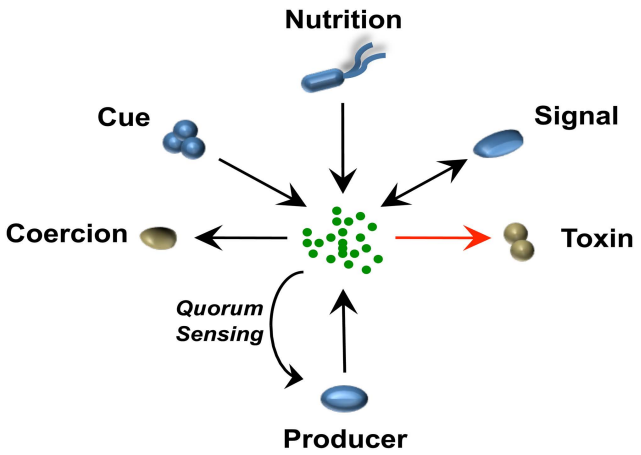

FIGURE 1 | A single chemical mediates different bacteria–bacteria interactions in a context of a polymicrobial community. The schematic represents how a single chemical released by one bacterial species can be perceived differently in a multispecies community. Microorganisms that benefit positively (blue) or negatively (brown) from the different interaction are represented. Arrows indicate direction of evolved pathway, e.g., an organism evolves to "sense" a cue or in some case can use an antibiotic as a nutritional source (Dantas et al., 2008). In the case of signaling and interspecies signaling, pathways from both producer and receiver cells have co-evolved to the benefit of both microorganisms. In the case of coercion or toxic interactions such as those exhibited by antibiotics, only the producer benefits from the interaction. When the producer itself detects the signal, this would be a case of quorum sensing or cell–cell signaling. In the case of antibiotics, the producer usually co-expresses resistance pathways and these can be co-expressed without a sensing circuit but often, such as with many lantibiotics, they are detected by the cell and respond in a classical quorum sensing feedback loop.

genetic mutations. The inability of cheaters to either produce and/or perceive a signal may abolish the bidirectional cooperative interaction. In a situation where the response toward a signal leads to the production of a protease allowing the degradation of a particular substrate for nutritional purposes, cheaters impaired in either the production or the reception of a signal will differentially impact the cooperative interaction. In fact, cheaters get a direct competitive advantage by avoiding the metabolic cost of producing a signal or responding to it and their selfish behavior allows them to benefit without being cooperative. However, some systems may have co-regulated pathways that help to control cheaters (Dandekar et al., 2012).

BIOACTIVITY AND ANTIBIOTIC RESISTANCE ARE DRIVEN BY ANTIBIOTIC CONCENTRATIONS

Antibiotics are generally known for their antimicrobial properties by which they either kill (bactericidal) or inhibit bacterial growth (bacteriostatic). Their concentrations are highly variable in natural communities and bacteria have evolved mechanisms to respond accordingly. Although their antimicrobial properties have been demonstrated in both *in vitro* and *in vivo* settings, the biological roles of antibiotics and antibiotic resistance in natural environments are still undefined.

To first differentiate the biological responses induced by antibiotics, here we represent their bioactivities from a receiver bacterium's perspective on a large concentration spectrum (**Figure 2**). From high to low concentrations, antibiotics act as either toxins, stress inducers, or as cues/coercions, respectively. Among interactions mediated by subinhibitory antibiotics, receiver bacteria can interestingly induce mechanisms leading to antibiotic resistance or tolerance (**Figure 2**). How antibiotics become stress inducers or cues/coercions will further be discussed in the following sections, but before we will briefly describe the toxin-like behavior of antibiotics and the impact of antibiotic resistance on the biological response exhibited by bacteria upon antibiotic exposure.

At concentrations superior to or near the minimal inhibitory concentration (MIC), antibiotics behave like toxins on susceptible bacterial cells. However, although the understanding of resistance mechanisms is well-characterized, molecular mechanism(s) induced by lethal concentrations of antibiotics is an area of research where our fundamental understanding is still very limited (Kohanski et al., 2010). Recent studies suggested that antibiotic-induced cell death was associated with increased production of radical oxygen species (ROS) such as hydroxyl radicals, superoxide, and hydrogen peroxide for bactericidal antibiotics belonging to the families of quinolones, β-lactams, and aminoglycosides (Dwyer et al., 2007, 2009; Kohanski et al., 2007, 2008, 2010). Although antibiotic ROS-mediated killing is highly possible in aerobic conditions, the proposed model is however, oxygen-dependent (Hassett and Imlay, 2007) and other mechanisms must operate in oxygen-poor environments such as those found in biofilm populations (Stewart and Franklin, 2008).

Antibiotic resistance mechanisms and the antimicrobial nature of antibiotics are often associated as a cause and effect phenomenon. The presence of resistance genes in bacteria with antibiotic biosynthesis genetic loci (Benveniste and Davies, 1973; Walker and Skorvaga, 1973; Davies and Benveniste, 1974) is an obvious self-protective strategy (Martinez, 2009a,b; Davies and Ryan, 2012; Wright and Poinar, 2012). It has therefore been widely accepted that antibiotic resistance determinants have specifically evolved to tolerate the lethal activity of antibiotics (Martinez, 2009a,b), but experimental data to fully support this thesis are still lacking (Davies and Ryan, 2012). Antibiotics are mainly present at non-lethal concentrations in the environment (Martinez, 2009a,b), therefore antibiotic resistance determinants are likely involved in response mechanisms other than those required when receiver bacteria are exposed to lethal concentrations. Similar to the antibiotic concentration-dependent response (**Figure 2**), antibiotic resistance would also impact receiver bacteria in an antibiotic dose-dependent manner. The presence of an antibiotic resistance mechanism would shift the spectrum of responses to an antibiotic to higher concentrations. The resistance would lower the effective concentration of antibiotics at the target site (**Figure 3A**). Exceptions to this would occur if there were secondary target sites for the antibiotics that mediate other responses. At toxic concentrations, resistance would function in the conventional protective role and allow receiver bacteria to avoid the antibiotic toxicity by blocking death or growth arrest. At subinhibitory concentrations, antibiotic resistance genes would shift the effective antibiotic concentration required for inducing the biological responses (stress inducers, coercion, and cues) of receiver

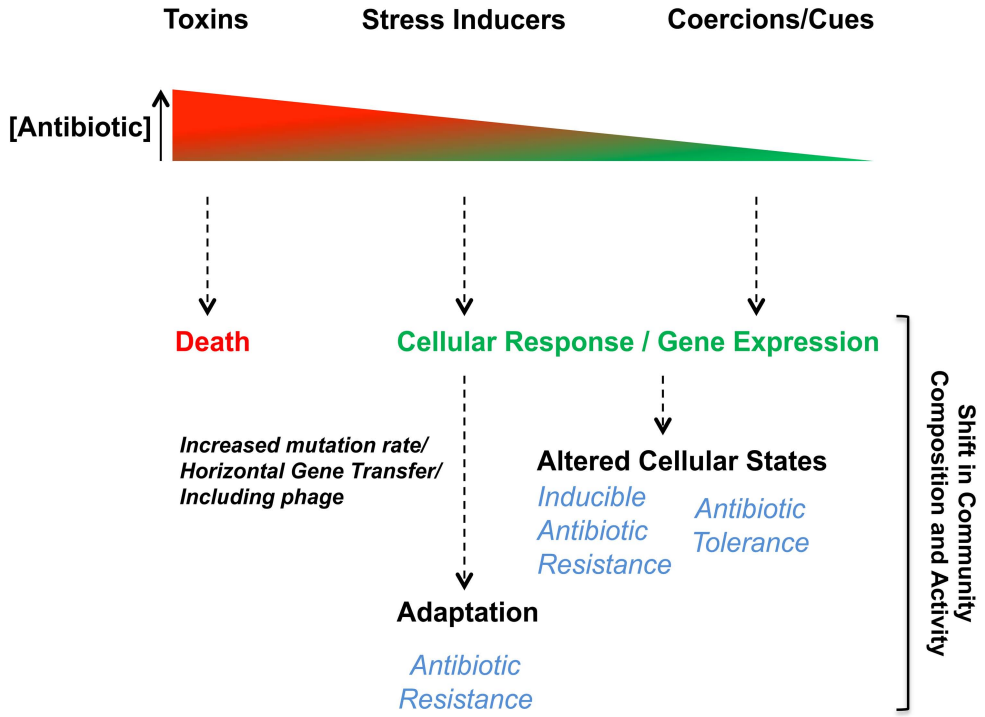

FIGURE 2 | Biological responses toward antibiotics are concentration-dependent. Bacterial interactions mediated by antibiotics induce biological responses in receiver bacteria in a dose-dependent manner. The antimicrobial behavior (toxin) of antibiotics occurs when their concentrations is high leading to bacterial death or growth arrest in susceptible receiver cells. At lower concentrations, subinhibitory, antibiotics can act as stress inducers, coercions or be sensed as cues. Biological responses induced in receiver bacteria when antibiotics are at subinhibitory concentrations can affect various cellular responses or alter gene expression leading to different adaptive responses impacting antibiotic resistance/tolerance.

bacteria (Goh et al., 2002; Yim et al., 2007; Mesak et al., 2008; Mesak and Davies, 2009). The displacement of the response curve to subinhibitory concentrations of antibiotics due to the presence of resistance determinants (**Figure 3A**) could therefore establish a chemical "*arms race*" between producer and receiver bacteria independent of lethal effect. In the context of bacterial communities, the displacement of the response curve for a single strain will modify its own behavior, which may in return shift the community composition or activity. Stress induction, coercion, and detection of cues are selectable phenotypes that can be tuned to meet the conditions of natural environments. Selections that reduce stress induction and coercion could be contributing factors in the evolution of the "cryptic resistome" (Wright, 2007); genes that are normally expressed at low levels or have low specific activity that do not confer resistance to the toxic effects of antibiotics at higher concentrations. These may be resistance genes tuned to environments where there are lower concentrations of these bioactive molecules.

In natural environments, antibiotics are likely present in a gradient of concentrations while receiver organisms may also be heterogeneous. Even in a homogeneous clonal population, cells may be present in different physiological states and the presence of antibiotic tolerance and resistance mechanisms will shift the response curves for individual cells within the population (**Figure 3B**). In the context of chemical interactions within natural communities, antibiotics released by emitter bacteria at lethal concentrations for the receiver cells would not be considered as a signaling event, but rather coercion at the extreme. Coercion can occur at subinhibitory concentrations and one bacterial cell may use chemicals to manipulate another. If the receiver cell possesses the corresponding antibiotic resistance determinant, receiver bacteria will be less susceptible to chemical manipulation and the overall benefit or harm of the interaction will therefore be reduced.

Outside of the well-mixed homogeneous environments of laboratory cultures, antibiotic effects on bacterial populations will be heterogeneous. For any bacteria, the response to a specific antibiotic will be concentration-dependent (**Figure 2**) and the active concentration for a particular response will be shifted higher by resistance mechanisms (**Figure 3A**). In natural environments, a population will be expected to exhibit a heterogeneous response because of gradients of antibiotic concentration and heterogeneity in the responsiveness of different cells in the population (**Figure 3B**). These differences in cellular responsiveness may be due to genetic heterogeneity and/or through differences in physiological states of different cells in the population.

ANTIBIOTICS AS STRESS INDUCERS

Antibiotics at subinhibitory concentrations can act as stress inducers or cues/coercion on receiver bacteria (**Figure 2**). When behaving as stress inducers, antibiotics often induce the SOS stress response, which is also associated with various antibiotic resistance mechanisms. The following section will mainly

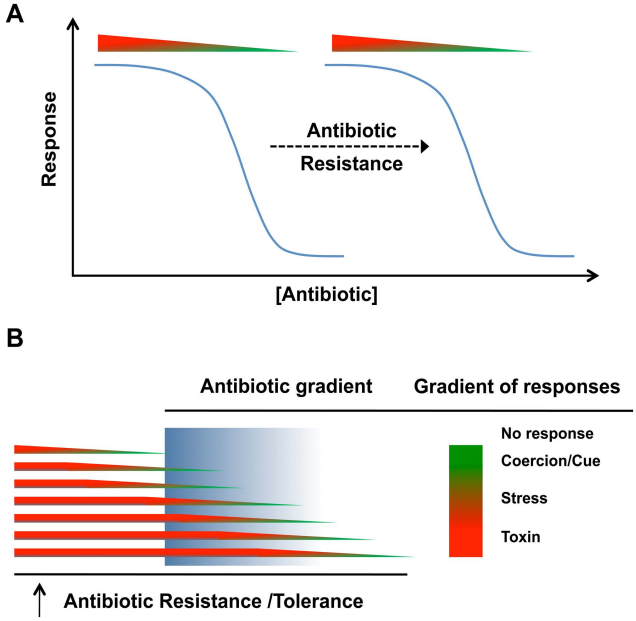

FIGURE 3 | Biological role(s) of antibiotic resistance in natural communities. (A) The response of bacterial cells to antibiotic whether it is bactericidal/bacteriostatic or subinhibitory, the biological response (as a cue or coercion) will be shifted by antibiotic resistance. **(B)** In natural environments, differences in physiological states can impact the expression of antibiotic resistance or tolerance mechanisms. Antibiotic concentrations in these environments may also be distributed as gradients, meaning that the population will not necessarily respond uniformly.

highlight some of the main resistance mechanisms impacted by the induction of the SOS response in bacteria upon antibiotic exposure.

Bacteria possess multiple survival mechanisms to cope with exogenous stresses and the SOS response is the main general stress response induced by bacteria in these situations (Galhardo et al., 2007; Blazquez et al., 2012; Poole, 2012b). The SOS stress response is typically induced upon DNA damage caused by extracellular stresses such as bacterial cell exposure to UV light or antibiotics (Erill et al., 2007; Janion, 2008; Butala et al., 2009). It is characterized by a well-coordinated global response initiating inhibition of cell division and induction of DNA repair, recombination, and mutation (Erill et al., 2007; Janion, 2008; Butala et al., 2009). In most bacterial species, the RecA and LexA proteins govern the response, which is conserved across bacterial phyla with a few exceptions where the LexA repressor protein homolog is absent, such as in *Streptococcus* species (Erill et al., 2007). Upon DNA damage, RecA stimulates cleavage of the LexA repressor leading to the global response involving more than 40 SOS-regulated genes (Courcelle et al., 2001; Erill et al., 2007; Janion, 2008; Butala et al., 2009). For a more comprehensive description of the SOS stress response, we refer the reader to consult some of these reviews that have thoroughly discussed the topic (Erill et al., 2007; Janion, 2008; Butala et al., 2009).

Fluoroquinolones and quinolones are broad spectrum antibiotics, for which resistant mechanisms have quickly emerged (Ruiz, 2003). They inhibit DNA gyrase leading to double-stranded DNA breaks and consequently induction of the SOS stress response (Urios et al., 1991; Dwyer et al., 2007; Yim et al., 2011). This induction usually occurs within a particular window of antibiotic concentrations (Piddock and Wise, 1987). In addition to fluoroquinolones or quinolones, bactericidal β-lactam and aminoglycoside antibiotics mediate bacterial killing by stimulating the production of ROS (Dwyer et al., 2007, 2009; Kohanski et al., 2007, 2008, 2010), which are themselves potent DNA damaging molecules (Farr and Kogoma, 1991). Consequently, all antibiotics mediating the production of ROS would therefore have the potential to induce the SOS stress response. Interestingly, fluoroquinolone and β-lactam antibiotics were shown in multiple studies to induce the SOS stress response in *Escherichia coli* while aminoglycosides failed (Ysern et al., 1990; Miller et al., 2004; Baharoglu and Mazel, 2011; Poole, 2012b). The intimate relationship between ROS-mediated killing and SOS-induction by bactericidal antibiotics requires more investigations to explain these discrepancies, but it does suggest that the stimulation of ROS production upon antibiotic exposure may be an indirect effect. Parallel mechanisms to the SOS response may exist for aminoglycosides to kill bacteria in a ROS-dependent manner or concentrations must be lethal to induce the SOS response and not subinhibitory. Other antibiotic classes represented by trimethoprim, ceftazidime, and sulfamethoxazole are also strong inducers of the SOS stress response in *E. coli* (Blazquez et al., 2012).

The SOS stress response is widespread among bacteria (Erill et al., 2007), but differences in the antibiotic SOS-induction profiles have been observed between species. For example, subinhibitory concentrations of tetracycline, chloramphenicol, and aminoglycosides induce the SOS response of *Vibrio cholerae*, while these antibiotics have no impact in *E. coli* (Baharoglu and Mazel, 2011). Despite the strong similarities between SOS systems across Gram-negative bacteria and the genetic relatedness of *E. coli* and *V. cholerae*, these disparities suggest that antibiotics may not necessarily induce the SOS response directly from DNA damage, but rather via upstream pathways or targets of the SOS response (Aertsen and Michiels, 2006) that could potentially differ between bacterial species. These differences between bacterial species may reflect the evolutionary selective pressure on the different bacteria with specific features reflecting conditions of their natural environments.

The induction of the SOS response is often essential for bacterial survival in stressful environments and associated with genetic responses that indirectly alter antibiotic resistance by increasing mutation rate, horizontal gene transfer, and prophage induction (Erill et al., 2007; Janion, 2008; Butala et al., 2009; Dwyer et al., 2009). Several studies have shown that induction of the SOS response by various antibiotics like fluoroquinolones, β-lactams, trimethoprim, tetracycline, chloramphenicol, rifampicin, and aminoglycosides increased the mutation frequency in different bacterial species (Ysern et al., 1990; Gillespie et al., 2005; Henderson-Begg et al., 2006; Cortes et al., 2008; Mesak and Davies, 2009; Baharoglu and Mazel, 2011; Yim et al., 2011). Induction of competency by fluoroquinolones leading to horizontal gene transfer with the potential to acquire new antibiotic resistance determinants has recently been reviewed (Charpentier et al., 2012). Furthermore, antibiotics inducing the SOS response can promote

bacterial genetic diversity via homologous recombination, phage release, and transfer of integrons or conjugative elements (Matic et al., 1995; Beaber et al., 2004; Cirz et al., 2007; Hocquet et al., 2012) all involved in the movement of mobile DNA like antibiotic resistance genes.

The induction of the SOS response is critical and relevant to our understanding of antibiotic-mediated interactions on the overall impact of antibiotic resistance in bacterial populations. Bacteria in most environments including those affected during therapeutic treatments can potentially encounter subinhibitory concentrations of antibiotics inducing the SOS stress response. Therefore, a better understanding of SOS-associated behaviors linked to antibiotic resistance traits may result in alternative approaches to control their spread.

ANTIBIOTICS AS CUES

Various antibiotics at subinhibitory concentrations induce biological responses on receiver bacteria that are non-stress-related and frequently affect pathways of primary metabolism (Goh et al., 2002; Tsui et al., 2004; Davies et al., 2006; Yim et al., 2006, 2007, 2011; Blazquez et al., 2012). Here we highlight examples demonstrating bacterial interactions mediated by antibiotics sensed as cues, which can subsequently impact the antibiotic resistance profile of receiver bacteria. These inducible responses can directly target mechanisms leading to specific antibiotic resistance or indirectly impact tolerance toward various antibiotics.

Many bacteria can sense specific antibiotics in their environment and subsequently induce the corresponding resistance mechanisms. Tetracycline and vancomycin are the two best-studied examples. Tetracycline resistance has been attributed to classical resistance mechanisms including efflux strategies, target site access (TetM and TetO), and chemical inactivation (TetX; Nelson and Levy, 2011). The regulation of some tetracycline resistance determinants is under the control of tetracycline repressor protein (TetR), which has been thoroughly reviewed elsewhere (Hillen and Berens, 1994; Berens and Hillen, 2003; Ramos et al., 2005; Nelson and Levy, 2011). Briefly, TetR has DNA binding domains targeting operators of tetracycline resistance genes. Once bound to the operator region of a target gene, TetR acts as a transcriptional repressor for which the repression can be relieved by the interaction of tetracycline with TetR (Hillen and Berens, 1994; Berens and Hillen, 2003; Ramos et al., 2005). Therefore, when tetracycline is present, the repressive function of TetR is abolished and transcription of the tetracycline resistance gene can occur normally.

In the case of vancomycin, modification of the target (peptidoglycan – D-Ala-D-Ala C-terminus) is the primary mechanism of resistance (Courvalin, 2006). Six types of resistance to vancomycin (VanA, B, C, D, E, and G) were reported in *Enterococcus* species (Courvalin, 2006). Interestingly, four of these operons involved in vancomycin resistance (VanA, B, E, and G) are directly inducible by vancomycin, while VanG and C types are constitutive (Courvalin, 2006). Vancomycin is sensed by a two-component regulatory system that positively activates expression of resistance genes in response to vancomycin. Although the basic regulation differs between tetracycline and vancomycin resistance genes, the inducible nature of their specific resistance pathways demonstrate that antibiotics have the ability to directly induce targeted and specific resistance mechanisms.

Although subinhibitory concentrations of antibiotics like tetracycline and vancomycin can induce their own resistance mechanisms, other biological responses have the potential to indirectly impact antibiotic tolerance as well. It was recently demonstrated that subinhibitory concentrations of β-lactam antibiotics induce the autolysin-dependent release of extracellular DNA (eDNA) by *Staphylococcus aureus*. This affects biofilm formation and autoaggregation (Kaplan et al., 2012), two growth protective mechanisms that will be discussed in the following section. Interestingly, eDNA that is part of the extracellular matrix of *P. aeruginosa* biofilms induces tolerance against aminoglycosides by chelating cations (Mulcahy et al., 2008). Additionally, subinhibitory concentrations of antibiotics like vancomycin, tetracycline, azithromycin, and ampicillin induce the expression of *P. aeruginosa* virulence-associated genes leading to increased secretion of phenazines and rhamnolipids (Shen et al., 2008). Pyocyanin, one of the four *P. aeruginosa* phenazines (Price-Whelan et al., 2006) was recently showed to induce eDNA release in *P. aeruginosa* biofilms through hydrogen peroxide (H_2O_2) mediating cell lysis (Das and Manefield, 2012). These examples provide evidence that in multispecies communities, subinhibitory antibiotics can lead to a series of sequential responses leading to antibiotic tolerance (**Figure 4**).

These are a few examples demonstrating that sensing of subinhibitory concentrations of antibiotics as cues can trigger direct or indirect mechanisms, for which receiver bacteria will subsequently have the ability to resist or tolerate lethal concentrations of antibiotics. In microbial communities, the response of one organism may lead to induction of antibiotic resistance/tolerance in other bacteria.

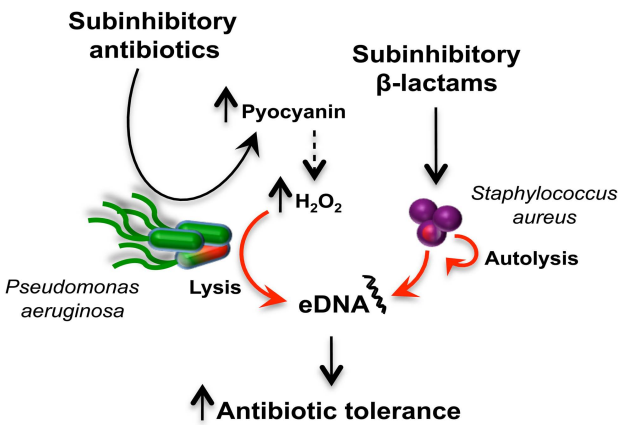

FIGURE 4 | Antibiotic induction of extracellular DNA (eDNA) release in a multispecies community and antibiotic tolerance. The schematic represents how two bacterial species, *P. aeruginosa* (green) and *S. aureus* (purple), respond to subinhibitory antibiotics to release eDNA. Subinhibitory antibiotics induced the production of pyocyanin (Shen et al., 2008), which is associated with increased H_2O_2 levels responsible for cell lysis and DNA release (Das and Manefield, 2012). In *S. arureus*, exposure to subinhibitory β-lactam antibiotics induces release of eDNA via an autolysis-dependent mechanism (Kaplan et al., 2012). The release of eDNA by both bacteria can induce antibiotic tolerance in communities (Mulcahy et al., 2008).

ANTIBIOTICS AS INDUCERS OF GROWTH PROTECTIVE MECHANISMS

A significant portion of the antibiotic resistome is made of mobile resistance genes that are horizontally transferable between bacterial cells (D'Costa et al., 2007; Wright, 2007; Allen et al., 2010). Mobile resistance determinants as well as efflux pumps account for the majority of antibiotic resistance mechanisms, however, bacteria have evolved non-inherited and transient mechanisms to resist otherwise lethal antibiotic concentrations (Levin and Rozen, 2006). Environmental conditions can trigger various stress responses making bacterial cells transiently refractory to antibiotics. Bacterial stress responses as determinants of antibiotic resistance have become an emerging area of research. For a more comprehensive description of these we refer the reader to recent reviews that have thoroughly addressed the topic (Poole, 2012a,b). Briefly, bacterial exposures to environmental-related stresses like nutrient starvation/limitation (nutrient stress), ROS and reactive nitrogen species (oxidative/nitrosative stress), membrane damage (envelope stress), temperature (heat/cold stress), and ribosome disruption (ribosomal stress) have the potential to initiate bacterial responses leading to modified or enhanced tolerance toward the lethal action of antibiotics (Poole, 2012a,b).

Non-inherited and transient mechanisms are mainly attributed to two distinct processes: persistence and drug indifference (Levin and Rozen, 2006). While persistence occurs in subpopulations of slow or non-growing bacteria, drug indifference can be exhibited by the entire population (Levin and Rozen, 2006). Persister cells are generally considered to be responsible for bacterial survival following antibiotic treatments although heterogeneity within bacterial populations and reduced accessibility of the drug to some target cells also contribute. A comprehensive description of persister cells is beyond the scope of this review; therefore we refer the reader to recent reviews that have thoroughly discussed the topic (Lewis, 2007, 2010, 2012; Gerdes and Maisonneuve, 2012; Kint et al., 2012). Persisters are phenotypic variants within an isogenic bacterial population that can tolerate high concentrations of antibiotics. In contrast to drug-resistant cells, persisters can switch back to the wild-type antibiotic sensitive phenotype when reactivated (Lewis, 2007, 2010, 2012; Gerdes and Maisonneuve, 2012; Kint et al., 2012). This persister cell behavior has been directly observed (Balaban et al., 2004). Persisters are present both in planktonic bacteria and antibiotic-tolerant biofilms (Spoering and Lewis, 2001). Research emphasis in the field has largely focused on mechanisms involved in persister formation and mechanisms leading to their formation have been proposed such as stochastic processes (passive) to active inducible regulation (Kint et al., 2012). Stochastic switching has been proposed as an effective strategy for survival in unpredictable environments (Kussell and Leibler, 2005; Kussell et al., 2005). Dormancy is a passive mechanism involved in persister formation resulting from stochastic endogenous stress leading to growth arrest and the shutdown of bactericidal antibiotic targets making persisters multidrug-tolerant cells (Lewis, 2007, 2010, 2012). Experimental evidence to support the dormancy theory came from transcriptome analysis showing that genes involved in primary metabolism and energy production were down-regulated in persister cells (Keren et al., 2004; Shah et al., 2006). However, the dormancy model was challenge when persisters of *E. coli* were demonstrated to display some level of protein translation (Gefen et al., 2008).

Active mechanisms leading to the induction of persisters in bacterial populations differ in terms of target. After the identification of *hipA*, as the first persistence gene and coding for the toxin of the *hipAB* toxin–antitoxin module (TA; Moyed and Bertrand, 1983), many studies have shown that different TA modules were involved in bacterial persistence (Lewis, 2010, 2012; Gerdes and Maisonneuve, 2012; Kint et al., 2012). The induction of toxin genes in persister cells (Keren et al., 2004; Shah et al., 2006) or the overexpression of toxins leading to increased persistence (Keren et al., 2004; Shah et al., 2006; Harrison et al., 2009; Maisonneuve et al., 2011) supported the role of TA modules in bacterial persistence. Interestingly, the fluoroquinolone ciprofloxacin was shown to induce bacteria persistence via the TA module TisAB upon activation of the SOS response (Dorr et al., 2010). Consistently, lethal concentrations of ampicillin or ofloxacin induce the SOS stress response in persister cells (Kaldalu et al., 2004) while the SOS response confers persistence to fluoroquinolones (Dorr et al., 2009). Unrelated to TA modules, the extracellular chemical indole was recently shown as mechanism inducing persisters in *E. coli* populations (Vega et al., 2012). Interestingly, the latter represents a non-antibiotic mediated interaction leading to antibiotic tolerance; a topic that will be discussed in the following section.

Beside unicellular growth, bacteria can also adopt various types of multicellular growth that exhibit phenotypes different than their planktonic counterparts including antibiotic tolerance. Multicellular behaviors in bacteria include growth as biofilms, aggregates, and swarming. Biofilms are sessile bacterial cells encased within an extracellular matrix that are usually attached to a surface (Stewart and Franklin, 2008; Monds and O'Toole, 2009). Bacterial aggregates are described as unattached biofilm-like structures with the ability to move (Alhede et al., 2011; Haaber et al., 2012; Thornton et al., 2012) while swarming represents a type of motility exhibited by bacteria over semi-solid surfaces (Kearns, 2010). These multicellular behaviors were all associated with elevated tolerance to lethal concentrations of antibiotics when compared to their planktonic counterparts (Stewart and Costerton, 2001; Kim et al., 2003; Lewis, 2007; Overhage et al., 2008; Lai et al., 2009; Alhede et al., 2011; Haaber et al., 2012; Thornton et al., 2012). These studies demonstrate that multicellular assemblies in bacteria confer an advantage when facing antibiotics compared to planktonic cells. However, the growth phase of planktonic cells (i.e., logarithmic or stationary) can have a significant impact on their antibiotic tolerance profiles. For example, planktonic *P. aeruginosa* and *E. coli* cells were previously shown to exhibit greater levels of tolerance against bactericidal antibiotics (Evans et al., 1991; Spoering and Lewis, 2001; Bernier et al., 2013). In *Salmonella typhimurium* shifts in primary metabolic pathways have been associated with the induced antibiotic tolerance in swarm cells (Kim and Surette, 2003, 2004; Turnbull and Surette, 2008, 2010) and aggregates (White et al., 2010).

Antibiotic-mediated interactions impact multicellular behaviors and indirectly the antibiotic tolerance profile of these

populations. Several studies have showed that subinhibitory antibiotics induce biofilm formation (Hoffman et al., 2005; Frank et al., 2007; Haddadin et al., 2010; Mirani and Jamil, 2011; Subrt et al., 2011; Kaplan et al., 2012) and autoaggregation (Kaplan et al., 2012). Bacterial response to extracellular stresses (Poole, 2012a,b) may also be an important trigger of multicellular behaviors in bacteria (Kaplan, 2011), which are better adapted as a group due to their physiology to tolerate lethal antibiotics (Stewart and Franklin, 2008). In support of this hypothesis, a recent study showed that non-starving planktonic cells were generally more tolerant to bactericidal antibiotics than biofilms, but when these same bacterial cells were starved, therefore stressed, biofilm bacteria were significantly more resilient to antibiotics than their planktonic counterparts (Bernier et al., 2013). As one of the first responses to stress, the SOS response is significantly more induced in biofilm cells compared to their planktonic counterparts (Beloin et al., 2004; Bernier et al., 2013). The higher intrinsic level of SOS in biofilms may explain their increased mutation frequency compared to planktonic cells (Conibear et al., 2009). Altogether, the increased SOS-dependent mutation rate observed in biofilms may well explain the high level of genetic variants arising in biofilm populations in a RecA-dependent manner (Boles et al., 2004; van der Veen and Abee, 2011) with the potential to impact antibiotic resistance (Boles and Singh, 2008).

The unique physiology of multicellular behaviors such as biofilms and swarming bacteria may render these cells better adapted to respond and tolerate extracellular stresses such as otherwise lethal antibiotic concentrations, these states may be induced directly by subinhibitory antibiotics.

NON-ANTIBIOTIC SMALL MOLECULES AS MODULATORS OF ANTIBIOTIC TOLERANCE

We have highlighted previous studies demonstrating that bacteria can sense antibiotics as cues to mediate bacteria–bacteria interactions with the potential to induce resistance/tolerance when lethal concentrations are subsequently reached. These can induce antibiotic specific mechanisms (e.g., tetracycline, vancomycin) or more general mechanisms like biofilms induction. However, the induction of antibiotic resistance/tolerance via cues is not limited to antibiotics. Within natural communities, bacteria are continuously exposed to a variety of small molecules other than antibiotics. Among these bioactive metabolites, some have been shown to induce biological responses in bacteria leading to a change in the overall antibiotic tolerance profile.

Bacterial-derived extracellular metabolites such as indole, hydrogen sulfide (H_2S), and volatile ammonia were recently shown to impact the antibiotic tolerance profile of receiver bacteria (Lee et al., 2010; Bernier et al., 2011; Shatalin et al., 2011; Vega et al., 2012). Interestingly, all of these bioactive molecules have the ability to be soluble or volatile, however, only ammonia was studied under its gaseous phase (Bernier et al., 2011).

Indole, a tryptophan-derived aromatic heterocyclic organic compound, was recently reported to induce antibiotic resistance in *E. coli* (Lee et al., 2010; Vega et al., 2012). A community-based antibiotic resistance mechanism was demonstrated to occur via the release of the metabolite in continuous cultures of *E. coli* exposed to increasing levels of the norfloxacin quinolone (Lee et al., 2010). Briefly, under antibiotic stress, a few drug-resistant mutants arise and then release the metabolite indole that is sensed by the entire population allowing other less resistant isolates to survive (Lee et al., 2010). In this particular case, the overall population MIC is totally biased by a few resistant clones since the majority of isolates are sensitive (Lee et al., 2010). The altruistic behavior of drug-resistant isolates comes with a fitness cost, associated with the production of indole, that benefits the entire population (Lee et al., 2010). Increased antibiotic tolerance mediated by indole is proposed to result from induction of efflux pumps and oxidative stress protective mechanisms (Lee et al., 2010). In a different study, indole is proposed to directly induce persistence through the generation of persister cells (Vega et al., 2012). The generation of persisters by indole exposure is dependent on the phage-shock (Psp) and OxyR pathways (Vega et al., 2012). In other studies, indole was shown to promote the establishment of *E. coli* in dual-species cultures with *P. aeruginosa* by inhibiting production of pyocyanin and other *P. aeruginosa* virulence factors regulated by quorum sensing (Chu et al., 2012). This example represents an example of coercion in which *E. coli*-derived indole manipulates *P. aeruginosa* as a strategy to colonize and share a polymicrobial community. Conversely, over production of indole through the induction of ROS production by subinhibitory concentrations of antibiotics was shown to impair *E. coli* biofilm formation (Kuczynska-Wisnik et al., 2010).

Until recently, the production of extracellular H_2S by bacteria has mainly been considered as a toxic by-product of metabolism. However, bacterial-derived H_2S is protective against the lethal action of antibiotics in a ROS-dependent manner (Shatalin et al., 2011). It was also demonstrated that endogenous nitric oxide (NO) of Gram-positive bacteria was protective against oxidative stress-mediated killing by macrophages (Shatalin et al., 2008) and antibiotics (Gusarov et al., 2009). Interestingly, NO and H_2S act synergistically since the absence of one can be compensated for by the increased production of the other one upon antibiotic exposure (Shatalin et al., 2011). These studies demonstrate the biological relevance of bacterial gases in mediating bacterial interactions and their indirect impact on antibiotic resistance.

Ammonia, a general by-product of amino acid catabolism, was recently shown to modulate the antibiotic tolerance of neighboring bacterial cells (Bernier et al., 2011). Briefly, both Gram-negative and Gram-positive bacterial species were able to tolerate otherwise lethal concentrations of ampicillin and tetracycline upon exposure to biogenic volatile ammonia (Bernier et al., 2011). Conversely, sensitivity toward aminoglycoside antibiotics was increased in bacterial cells exposed to volatile ammonia (Bernier et al., 2011). Ammonia-mediated interactions between bacterial cells were shown to induce the intracellular levels of polyamines (Bernier et al., 2011). Consistently, addition of polyamines (spermidine and putrescine) could recapitulate the ammonia-mediated phenotype demonstrating that the modified antibiotic tolerance profile of receiver bacteria was fully dependent on the polyamine modulon upon ammonia exposure (Bernier et al., 2011). Interestingly, it was demonstrated in *Bacillus* spp. that biofilm formation could also be induced upon exposure to biogenic ammonia and polyamines were critical for normal biofilm development (Burrell et al., 2010; Nijland and Burgess, 2010).

The close relationship between ammonia sensing, polyamine induction, and biofilm formation demonstrate that bacterial interactions mediated by non-antibiotic molecules can modulate antibiotic tolerance not only locally, but also at a distance when ammonia is under its gaseous phase. Altogether, non-antibiotic-mediated interactions clearly demonstrate the complexity of dealing with antibiotic resistance/tolerance when bacteria are part of complex communities, making antibiotic treatments unpredictable.

METABOLITE-MEDIATED INTERACTIONS IN BACTERIAL COMMUNITIES AS MECHANISMS OF ANTIBIOTIC RESISTANCE EVOLUTION

Many infections are polymicrobial and as presented above, antibiotics and non-antibiotic metabolites can mediate interactions between organisms that may impact their efficacy as antimicrobials. The following describes a hypothetical scenario demonstrating how metabolite-mediated interactions may have the potential to alter the antibiotic resistance profile of an entire population and possibly leading to the spread of antibiotic resistance.

For this exercise, we chose a simple polymicrobial community including the pathogens *P. aeruginosa* and *S. aureus* as well as *Streptococcus* species belonging to the normal oropharyngeal microbiota. These bacterial species can simultaneously co-infect the lungs of cystic fibrosis (CF) patients (Sibley et al., 2008b, 2009, 2011) and dynamic interactions between these organisms have previously been demonstrated to alter *P. aeruginosa* virulence (Duan et al., 2003; Sibley et al., 2008a). In the scenario described below, we suggest that a single antibiotic therapy may affect the overall community structure and subsequently the antibiotic resistance of the community by initiating a cascade of inter-species interactions mediated by antibiotics and non-antibiotic metabolites (**Figure 5**).

Following an antibiotic treatment, many of the bacterial cells in the community will lyse releasing their intracellular content including eDNA and NO that could potentially induce antibiotic tolerance (Mulcahy et al., 2008; Gusarov et al., 2009). However, because of the population heterogeneity in terms of susceptibility and resistance/tolerance (persisters, stationary phase cells, aggregates, and biofilms) a large number of cells will die while some will survive. Over the course of the treatment, antibiotics will be present at different concentrations at different times and sites and therefore mediating different types of interactions with viable bacterial cells. At subinhibitory concentrations, antibiotics will induce different biological responses, for which antibiotic resistance/tolerance will subsequently be affected. In this particular polymicrobial community, β-lactams will induce the release of eDNA from *S. aureus* in an autolysin-dependent manner and

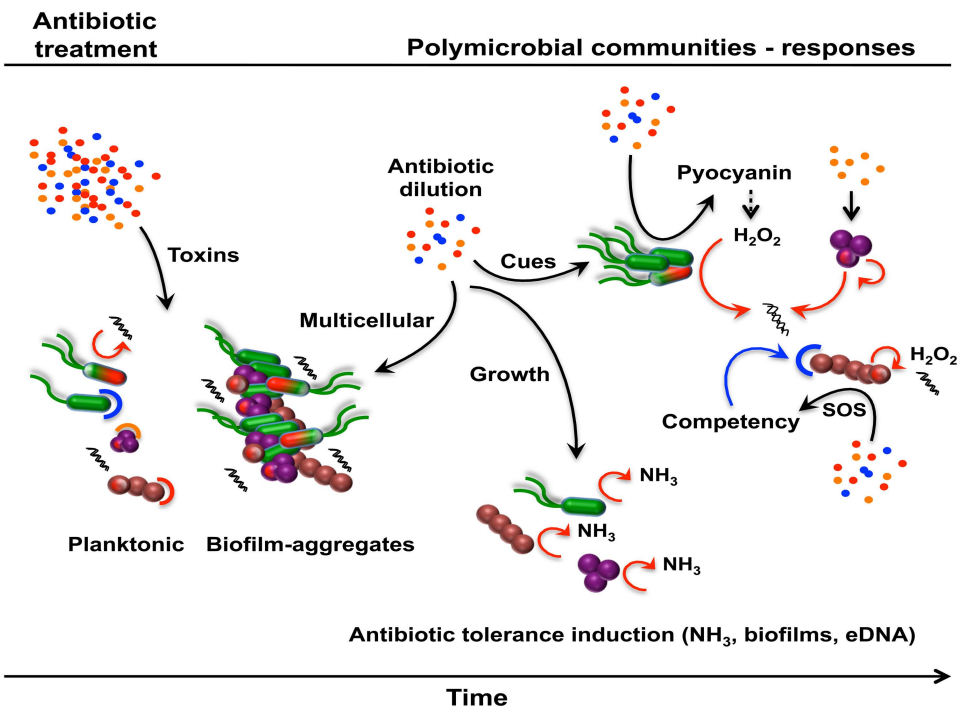

FIGURE 5 | Metabolite-mediated interactions modulating antibiotic resistance or tolerance in polymicrobial communities. Following an antibiotic treatment, natural communities will respond, adapt, and evolve through the establishment of dynamic interactions mediated by antibiotics and non-antibiotic metabolites. A simple polymicrobial community comprising *P. aeruginosa* (green), *S. aureus* (purple), and *Streptococcus* spp. (brown) is used to illustrate this hypothetical scenario. A proportion of the established community, made of multispecies biofilms and planktonic cells, will die and subsequently lyse upon antibiotic exposure, unless cells harbor the corresponding antibiotic resistance determinant (capped cells; red, blue, or orange corresponding to the respective antibiotic) or are part of multicellular structures (biofilms, aggregates). Subinhibitory antibiotics and non-antibiotic metabolites within the community can subsequently induce different antibiotic tolerance mechanisms leading to an altered community in terms of composition and antibiotic resistance/tolerance.

triggering biofilm formation as a growth protective mechanism (Kaplan et al., 2012). At the same time, subinhibitory concentrations of other antibiotics will induce the production of pyocyanin in *P. aeruginosa* (Shen et al., 2008) resulting in the generation of H_2O_2, cell lysis, and subsequent release of more eDNA (Das and Manefield, 2012). Streptococci bacteria are known to generate H_2O_2 causing cell lysis and the release of eDNA (Shen et al., 2008). The accumulation of eDNA, through antibiotic and non-antibiotic interactions, will chelate cations and primarily the reduced cation concentrations that are directly sensed by the cells leading to antibiotic tolerance (Mulcahy et al., 2008). In addition, the community would favor biofilms and aggregates as modes of growth (Kaplan et al., 2012), which are generally more tolerant to antibiotics.

The concentration of antibiotics will gradually decrease over time allowing the remaining viable bacterial cells to grow back. The CF lung is rich in amino acids (Palmer et al., 2007) and actively growing bacteria will release ammonia as a by-product of amino acid catabolism. Ammonia sensing by bacteria within the community will induce the synthesis of polyamines leading to increased tolerance against ampicillin and tetracycline (Bernier et al., 2011) and oxidative stress (Bernier et al., 2011; Johnson et al., 2012). Further, the CF lung itself is rich in extracellular polyamines (Grasemann et al., 2012), which could directly affect the antibiotic tolerance profile of the community (El-Halfawy and Valvano, 2012) independently of ammonia sensing. Further, the consequences of these responses may impact syntrophic and other metabolite-mediated interactions that can further alter community composition.

For bacterial species that are naturally competent, the abundance of eDNA may also represent an excellent source of antibiotic resistance genes. Subinhibitory antibiotics induce the SOS response and competence systems (Charpentier et al., 2012) in Streptococci leading to horizontal gene transfer and the acquisition of resistance genes. Thereafter, the new-acquired resistance gene will be transferred vertically through bacterial division resulting in a new drug-resistant Streptococci strain. Further, mutation rates are also increased in all bacteria upon antibiotic exposure leading to the generation of potential new drug-resistant mutants. Resistance mechanisms that chemically inactivate antibiotics also have the potential to reduce the concentration to subinhibitory levels for susceptible cells in the community.

Through various inducible mechanisms mediated by antibiotics and non-antibiotic metabolites, the overall composition of the community may change over time. This complex network of interactions is not reflected in standard antibacterial susceptibility and these processes likely contribute to the frequent failure of antibiotics to reduce the population of susceptible organisms in patients. This will be more likely to occur in polymicrobial infections or when pathogens are part of a normal host community such as in upper respiratory or gastrointestinal infections.

CONCLUSIONS AND PERSPECTIVES

The increase of antibiotic-resistant pathogens represents a very significant threat and challenge in the fight against infectious diseases. The complexity of the antibiotic resistome demonstrates that antibiotic resistance will always be a menace even for synthetic antibiotics. Fluoroquinolones represent a good example of synthetic drugs against which bacteria have quickly evolved resistance (Ruiz, 2003; D'Costa et al., 2006, 2007). More judicious use of antibiotics clinically and restricting non-medical applications may slow down the spread of drug-resistant bacteria and the emergence of new antibiotic-resistant pathogens, but the breadth of the antibiotic resistome and the capacity of microbes to rapidly evolve will make this an ongoing struggle. The chemical warfare that has been going on in microbial communities for hundreds of millions of years is something of a double-edged sword. Most antibiotics in use today have a microbial origin. Microbial secondary metabolites have also been a valuable source of drugs not restricted to just antibiotics. At the same time these communities have evolved complex resistance mechanisms that can rapidly spread from natural environments to the clinic.

Since most antibiotics are natural molecules involved in chemical interactions between bacteria in communities, it has become important to expand our understanding of the effects of these interactions on bacterial behaviors including antibiotic resistance. Because the presence of subinhibitory antibiotics can result in a phenotype, the responses are subject to evolution and natural selection, the same as toxic interactions. In this review, we have discussed and presented various scenarios on how antibiotics at subinhibitory concentrations have the potential to induce different biological responses leading to a general modification in the antibiotic resistance profiles of both environmental and host-associated bacteria. These non-lethal interactions can act as stress inducers or be sensed as cues by receiver bacteria. Activation of the SOS stress response by antibiotics appears to reduce the efficacy of antibiotic treatments and facilitate the evolution of resistance. Therefore, new therapeutic strategies targeting the SOS response may in return increase antibiotic efficacy. Blocking the LexA cleavage, therefore the SOS response, reduces the ability of *E. coli* to develop resistance toward ciprofloxacin and rifampicin both *in vivo* and *in vitro*, through mutations (Cirz et al., 2005). This suggests that suppressing the SOS response would inhibit mutation rate, which is an important downstream SOS-associated phenotype involved in bacterial evolution and antibiotic resistance.

Manipulating cellular physiology has the potential to enhance the efficacy of antibiotics even in resistant strains. A recent study explored this possibility and reported that an engineered bacteriophage targeting the SOS response network enhanced the killing efficacy of bactericidal antibiotics and survival in mice (Lu and Collins, 2009). The enhanced antibiotic killing by SOS-targeting phages was also effective against persister and biofilm bacteria (Lu and Collins, 2009).

The nature of the effect of volatile ammonia on antibiotic resistance is dependent on the class of antibiotic. While bacterial exposure to volatile ammonia induces tolerance against β-lactam and tetracycline antibiotics it increases the efficacy of aminoglycosides (Bernier et al., 2011). Interestingly, reactivation of the metabolism of *E. coli* persisters by the addition of various metabolites (glucose, mannitol, fructose, pyruvate) restored their sensitivity to aminoglycosides to a level comparable to non-persister cells (Allison et al., 2011). These two studies demonstrate the proof of concept that bacterial interactions occurring in natural communities via non-antibiotic molecules have the ability

to increase antibiotic efficacy. Non-antibiotic metabolites like ammonia or glucose could be considered as antibiotic potentiators in synergistic drug combination therapies. The combinations of antibiotics and non-antibiotic drugs were showed to enhance antimicrobial efficacy against multidrug-resistant bacteria in both *in vivo* and *in vitro* suggesting that synergistic drug combinations have therapeutic potentials (Ejim et al., 2011).

Furthering our understanding of bacterial interactions mediated by antibiotics and non-antibiotic molecules will be valuable in the development of new strategies to combat antibiotic resistance. Although significant findings have been made in this emerging area of research, we need to further expand our views of these dynamic interactions in microbial communities. The conventional approach of determining *in vitro* susceptibilities to isolated organisms has many limitations clinically and in polymicrobial infections, many community interactions can reduce antibiotic efficacy in addition to traditional resistance mechanisms. Understanding chemical interactions within microbial communities should include the role of antibiotics in these communities and mechanisms of resistance. This will provide new opportunities and strategies to accelerate the discovery of bioactive molecules. Finally, we have to continue our efforts toward our global understanding of these non-classical views of antibiotic-mediated resistance mechanisms as complementary and potential strategies to new drug development programs in order to control and adequately manage the spread of antibiotic resistance in the future.

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